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Method for assaying glycoprotein by treating samples with microbially derived proteases and oxidases

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Abstract (Basic): WO 200125475 A1

NOVELTY - Method for detecting and measuring glycoprotein comprises treatment of the sample with a protease, followed by an oxidase that produces hydrogen peroxide and detecting the presence or amount of component produced or consumed.

DETAILED DESCRIPTION - An INDEPENDENT CLAIM is also included for a kit for assaying glycoprotein containing a protease, oxidase and a reagent for measuring peroxide.

USE - Assaying for glycoproteins.

ADVANTAGE - This method uses HPLC (high performance liquid chromatography) and is simpler, cheaper and quicker than conventional methods using mass spectrometric analysis.

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Title Terms: METHOD; ASSAY; TREAT; SAMPLE; MICROBE; DERIVATIVE

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(54) 【発明の名称】 糖化蛋白質の測定方法

(57) 【要約】

【課題】 既存の酵素的方法とは異なる原理に基づく、簡単な操作で、短時間でしかも精度よく糖化蛋白質を測定する新規な方法を提供することにある。

【解決手段】 糖化蛋白質を含む試料をプロテアーゼで処理し、糖化蛋白質から糖化ペプチド、好ましくは $\alpha$ -糖化ペプチド、特に好ましくは $\alpha$ -糖化ジペプチドを遊離させ、これらの遊離した糖化ペプチドにオキシダーゼを作用させ、生成する過酸化水素を測定すること、又は遊離した糖化ペプチドをHPLCにより測定することにより、試料中の糖化蛋白質を測定する方法、および酵素的方法に用いる測定用試薬キットである。

## 【特許請求の範囲】

【請求項1】 試料をプロテアーゼで処理して、遊離した糖化ペプチドを、以下の(1)又は(2)の何れかの方法で測定することを特徴とする、試料中の糖化蛋白質の測定方法。

(1) 遊離した糖化ペプチドにオキシダーゼを作用させ、その作用による生成物または消費物を測定することにより、糖化ペプチドを測定する方法。

(2) 遊離した糖化ペプチドをHPLCを用いて測定する方法。

【請求項2】 プロテアーゼが、アスペルギルス属、サッカロミセス属又はバチルス属の微生物の生産するプロテアーゼから選ばれる1種以上のプロテアーゼである請求項1記載の糖化蛋白質の測定方法。

【請求項3】 オキシダーゼが、糖化ペプチドに作用して、過酸化水素を生成する作用を有することを特徴とするオキシダーゼである、請求項1又は2記載の糖化蛋白質の測定方法。

【請求項4】 糖化ペプチドが $\alpha$ -糖化ペプチドである、請求項1、2又は3記載の糖化蛋白質の測定方法。

【請求項5】  $\alpha$ -糖化ペプチドが $\alpha$ -糖化ジペプチドである請求項4記載の糖化蛋白質の測定方法。

【請求項6】  $\alpha$ -糖化ジペプチドがフルクトシルバリンヒスチジンである、請求項5記載の糖化蛋白質の測定方法。

【請求項7】 測定する生成物が過酸化水素である請求項1〜6いずれか1項記載の糖化蛋白質の測定方法。

【請求項8】 以下の成分を含むことを特徴とする、試料中の糖化蛋白質の測定用試薬キット。

(1) プロテアーゼ。

(2) 糖化ペプチドに作用して、過酸化水素を生成する作用を有するオキシダーゼ。

(3) 過酸化水素を測定するための試薬。

【請求項9】 糖化ペプチドが $\alpha$ -糖化ペプチドである、請求項8記載の試料中の糖化蛋白質の測定用試薬キット。

【請求項10】  $\alpha$ -糖化ペプチドが $\alpha$ -糖化ジペプチドである、請求項9記載の試料中の糖化蛋白質の測定用試薬キット。

【請求項11】  $\alpha$ -糖化ジペプチドがフルクトシルバリンヒスチジンである請求項10記載の試料中の糖化蛋白質の測定用試薬キット。

## 【発明の詳細な説明】

【0001】

【発明の属する技術分野】 本発明は、試料中の糖化蛋白質の測定方法、およびその測定方法に用いられる測定用試薬キットに関する。

【0002】

【従来の技術】 糖化蛋白質は、蛋白質が非酵素的にグリコシル化された蛋白質であり、糖、すなわちアルドース

(アルデヒド基を潜在的に有する単糖およびその誘導体)側のアルデヒド基と、蛋白質側のアミノ基が非酵素的に共有結合した結果、生成したものである。また、これらの糖化蛋白質は、反応中間体として生じたシッフ塩基がアマドリ転移を受けて形成されることから、いわゆるアマドリ化合物とも呼ばれる。

【0003】 糖化蛋白質は、生体内の血液などの体液や毛髪などの生体試料中に含有されている。血液中に存在する糖化蛋白質の濃度は、血清中に溶解しているグルコースなどの糖類の濃度に強く依存している。糖尿病状態では糖化蛋白質の生成が亢進しており、赤血球に含まれる糖化ヘモグロビンや血清中の糖化アルブミンの濃度は、過去の一定期間の平均血糖値を反映していることから、それらの糖化蛋白質を測定することは、糖尿病の症状の診断や症状管理に重要となっている。

【0004】 従来、糖化蛋白質を定量する方法として、例えば、高速液体クロマトグラフィーを用いる方法 (Chromatogr. sci., 10, 659 (1979))、硼酸を結合させた固体を詰めたカラムを用いる方法 (Clin. Chem., 28, 2088-2094 (1982))、電気泳動を用いる方法 (Clin. Chem., 26, 1598-1602 (1980))、抗原抗体反応を利用する方法 (JJCLA, 18, 620 (1993))、還元能をテトラソリウム塩を用いて比色定量する方法 (Clin. Chim. Acta, 127, 87-95 (1982))、チオバルビツール酸を用いて酸化後比色定量する方法 (Clin. Chim. Acta, 112, 197-204 (1981)) 等が知られる。現在、上記方法よりも、操作が簡単で、安価に、短時間で精度よく糖化蛋白質を測定する方法として、酵素的な方法が提案されている (特公平05-33997号公報、特開平11-127895号公報、WO97-13872号公報)。

【0005】 これらの酵素的な方法は、糖化蛋白質をプロテアーゼで分解し、遊離した糖化アミノ酸にフルクトシルアミノ酸オキシダーゼを作用させ、生成する過酸化水素を測定する方法である。この酵素的測定方法に用いられるオキシダーゼとして、コリネバクテリウム属菌の生産するオキシダーゼ (特公平5-33997号公報、特公平6-65300号公報)、アスペルギルス属菌の生産するオキシダーゼ (特開平3-155780号公報)、ギベラ属菌の生産するオキシダーゼ (特開平7-289253号公報)、フサリウム属菌の生産するオキシダーゼ (特開平7-289253号公報、特開平8-154672号公報)、ペニシリウム属菌の生産するオキシダーゼ (特開平8-336386号公報) さらには、ケトアミンオキシダーゼ (特開平5-192193号公報) などが開示されており、これらの酵素は、糖化アミノ酸に良く作用する。しかし、上記酵素は、ペプチドのアミノ基が糖化された糖化ペプチドに対しては作用しない。

【0006】 現在、糖尿病診断の指標として用いられている糖化蛋白質としては、蛋白質中の内部リジン残基の

$\epsilon$ -アミノ基が糖化されたもの（例えば糖化アルブミン）や蛋白質のアミノ末端のアミノ酸の $\alpha$ -アミノ基が糖化されたもの（例えば糖化ヘモグロビン（HbA1c））など、種々の糖化蛋白質が挙げられる。しかし、現在、対象とする糖化蛋白質によっては、既存のプロテアーゼを用いても、糖化蛋白質を分解して、定量的に糖化アミノ酸を遊離することはできず、さらに、現在用いられている上記したフルクトシルアミノ酸オキシダーゼは、遊離の糖化アミノ酸に対し高い反応性を有しているものの、糖化ペプチドにはほとんど作用しないため、上記酵素的方法は、必ずしも精度の良い方法とは言えない。

【0007】例えば、糖化ヘモグロビン（HbA1c）は、ヘモグロビン $\beta$ -サブユニットのアミノ末端のアミノ酸の $\alpha$ -アミノ基が糖化されたものであるが、この糖化蛋白質に各種のプロテアーゼを作用させても、 $\alpha$ -糖化アミノ酸（アミノ酸の $\alpha$ -アミノ基が糖化されたもの）を遊離させることはできない。そのため、前述のフルクトシルアミノ酸オキシダーゼを用いても、糖化ヘモグロビン（HbA1c）を測定することができない。

【0008】現在、この糖化ヘモグロビン（HbA1c）を測定する方法として、この糖化ヘモグロビンを、そのまま直接、エレクトロスプレーイオン化質量分析で測定する方法（臨床検査、42,340-343,（1997））や、糖化ヘモグロビンに、エンドプロテイナーゼGlu-Cを作用させ、遊離した $\beta$ -サブユニット由来の $\alpha$ -糖化ヘキサペプチド（ヘキサペプチドのアミノ末端のアミノ酸の $\alpha$ -アミノ基が糖化されたもの）を逆相高速液体クロマトグラフィーで分取し、マスマスペクトロメトリー分析に供して、その含有率を求めることにより、測定する方法（Clin. Chem., 43, 1994-1951（1997））等が提案されている。しかし、これらの方法は、何れも高感度の高価な測定装置を必要とし、操作も複雑で、費用もかかり、長時間を必要とする。

【0009】

【発明が解決しようとする課題】本発明は、このような従来の糖化蛋白質の測定方法が有する欠点を克服し、既存の酵素的方法とは異なる原理に基づく、簡単な操作で、安価に、短時間でしかも精度よく糖化蛋白質を測定する新規な方法を提供することにある。

【0010】

【課題を解決するための手段】本発明者等は、前記課題解決のために鋭意研究を重ねた結果、ある種のプロテアーゼ処理により、糖化蛋白質から一定数のアミノ酸残基を有する $\alpha$ -糖化ペプチド（ペプチドのアミノ末端のアミノ酸の $\alpha$ -アミノ基が糖化された糖化ペプチド）、特に $\alpha$ -糖化ジペプチド（ジペプチドのアミノ末端のアミノ酸の $\alpha$ -アミノ基が糖化された糖化ジペプチド）が、効率よく遊離すること、又、微生物の生産するフルクトシルアミノ酸オキシダーゼを改変した酵素が、上記の遊

離 $\alpha$ -糖化ペプチド、特に、 $\alpha$ -糖化ジペプチドに特異的に作用し、過酸化水素を生成することを見いだした。さらに、糖化蛋白質より遊離した $\alpha$ -糖化ペプチドをHPLCや上記オキシダーゼを用いることにより測定できること、その結果、糖化蛋白質を、簡単な操作で、短時間に、精度よく測定できることを見出し、これらの知見に基づき本発明を完成するに至った。

【0011】すなわち本発明は、試料をプロテアーゼで処理し、糖化蛋白質から糖化ペプチド、好ましくは $\alpha$ -糖化ペプチド、特に好ましくは $\alpha$ -糖化ジペプチドを遊離させ、これらの遊離した糖化ペプチドにオキシダーゼを作用させ、生成する過酸化水素を測定すること、又は、遊離した糖化ペプチドをHPLCにより測定することにより、試料中の糖化蛋白質を測定する方法、および酵素的方法に用いる測定用試薬キットである。

【0012】

【発明の実施の形態】以下本発明を詳細に説明する。本発明における糖化蛋白質は、前述したように、蛋白質がグルコースなどのアルドースと非酵素的に結合し、生成したものであれば如何なるものでも良い。例えば、生体由来の糖化蛋白質としては、糖化アルブミン、糖化ヘモグロビン（HbA1c）などがあり、本発明は、例えば、糖化ヘモグロビン（HbA1c）などの測定に好適に用いられる。さらに、糖化蛋白質は、食品一般、例えば、ジュース、キャンデー、調味料、粉末食品などにも含まれている。本発明の糖化蛋白質を含有する試料としては、上記糖化蛋白質を含有する試料であれば如何なるものでもよく、例えば、生体内では、血液、唾液などの体液や毛髪など、そして上記食品などが挙げられる。これらの試料は、そのままあるいは濾過、透析処理などの後に測定に供してもよく、また、例えば、測定すべき糖化蛋白質を、適宜、濃縮、抽出、さらには水、緩衝液などで希釈しても良い。

【0013】本発明においては、先ず、プロテアーゼを用い、糖化蛋白質より糖化ペプチドを遊離させる。尚、本発明で言う、プロテアーゼとは、通常のプロテアーゼ活性、及びペプチダーゼ活性を有する酵素を言う。用いるプロテアーゼは、上記糖化蛋白質に作用し、糖化ペプチドを遊離する能力を有する酵素であれば、如何なる酵素でも用いることができ、目的の糖化蛋白質の種類に応じ、好適なものを適宜選択することができる。例えば、プロテイナーゼK、プロナーゼE、サーモリシン、ズブチリシン、カルボキシペプチダーゼB、カテプシン、カルボキシペプチダーゼ、エンドプロテイナーゼGlu-C、パピイン、アミノペプチダーゼなどのプロテアーゼやペプチダーゼが挙げられる。本発明では、後述する本発明に用いられるオキシダーゼが作用しやすい糖化ペプチドを効率よく遊離する能力を有するプロテアーゼが望ましい。特に $\alpha$ -糖化ジペプチドを効率よく遊離するプロテアーゼとして、アスペルギルス属菌由来のプロテア

一ゼ、例えば、「モルシン」、「AOプロテアーゼ」、「ペプチダーゼ」(以上(株)盛進、販売)、サッカロミセス属由来のカルボキシペプチダーゼY、バチルス属菌由来のプロテアーゼ、例えば、プロチンP(大和化成(株)、販売)などのプロテアーゼを含むものが、特に好適に用いられる。上記プロテアーゼは、単独で用いても、また2種以上を組み合わせて用いてもよい。

【0014】試料の処理条件は、用いるプロテアーゼが、測定対象となる糖化蛋白質に作用し、糖化ペプチドを短時間に効率よく遊離する条件であれば、如何なる条件でもよい。用いられるプロテアーゼの量は、試料中に含まれる糖化蛋白質の含量や処理条件などにより適宜選択されるが、例えば、一例として、アスペルギルス属菌由来のプロテアーゼ(例えば、モルシン、(株)盛進、販売)を0.5~50mg/mL、好ましくは1~20mg/mL加える。さらに必要により適宜他のプロテアーゼを加えてもよい。プロテアーゼで処理するときのpHは、無調整でもよいが、使用するプロテアーゼの作用に好適なpHとなるように、例えば、適当なpH調整剤、例えば塩酸、酢酸、硫酸、水酸化ナトリウム、水酸化カリウムなどにより、pH2~9、好ましくはpH3~8に調整してもよい。処理温度は、例えば、20~50℃で行なってもよいし、用いる酵素によっては、より高温域の45~70℃で行なっても良い。このときの処理時間は、糖化蛋白質を分解するのに充分な時間であればよく、1~180分間、好ましくは2~60分間で行なうことができる。得られる処理液は、そのまま、あるいは必要により適宜、加熱、遠心分離、濃縮、希釈などをしてよい。

【0015】試料をプロテアーゼで処理して得られる、本発明の遊離した糖化ペプチドは、糖化蛋白質を上記プロテアーゼで処理して得られる糖化ペプチドであって、後述する本発明に用いられるオキシダーゼが作用しやすい糖化ペプチドであれば、如何なるものも含まれるが、好ましくは、 $\alpha$ -糖化ペプチドであり、例えば、ペプチドのアミノ酸数が2~6の短鎖の $\alpha$ -糖化ペプチドなどが挙げられる。特に好ましくは、 $\alpha$ -糖化ジペプチド、例えば、フルクトシルバリルヒスチジン(以下、フルクトシルVal-His、又はFru-Val-Hisと略す)などが挙げられる。また、生体中や食品中には、糖化蛋白質がそれぞれ生体中や食品の製造過程で、既に分解されて遊離した糖化ペプチドとなったものや、蛋白質などが分解されて、遊離したペプチドに糖が結合してきた糖化ペプチド等も含まれており、これらも本発明の遊離した糖化ペプチドに含まれる。

【0016】次に、上記した糖化ペプチドを測定する。糖化ペプチドを測定することが可能であれば、如何なる方法でも本発明に用いることができる。簡単な操作で、安価に、短時間でしかも精度よく糖化ペプチドを測定するための好ましい方法として、例えば、オキシダーゼを

作用させる方法やHPLCを用いる方法などを挙げることができる。

【0017】本発明のオキシダーゼを作用させる方法について説明する。上記糖化ペプチドにオキシダーゼを作用させ、その作用による生成物または消費物を測定することにより、酵素的方法で糖化ペプチドを測定する。本発明に用いられるオキシダーゼとしては、糖化ペプチド、好ましくは $\alpha$ -糖化ペプチド、特に好ましくは $\alpha$ -糖化ジペプチドなどの短鎖の $\alpha$ -糖化ペプチドに特異的に作用して、過酸化水素を生成する反応を触媒する酵素(以下、本発明のオキシダーゼと言う)であれば如何なる酵素でも用いることができる。例えば、糖化ペプチドオキシダーゼなどの新規なオキシダーゼが挙げられる。

【0018】一般には、上記本発明のオキシダーゼを生産する微生物を自然界より探索して得ることができるし、さらに動物や植物起源の本発明の酵素を探索して得ることもできる。さらに、探索して得られたこれらの酵素を遺伝子組換え技術を用いて得たものでも好適に用いることもできる。一方、既知のフルクトシルアミノ酸オキシダーゼなどを改変することにより、本発明のオキシダーゼを得ることもできる。例えば、既知のフルクトシルアミノ酸オキシダーゼなどとしては、先に述べたコリネバクテリウム属菌の生産するオキシダーゼ(特公平5-33997号公報、特公平6-65300号公報)、アスペルギルス属菌の生産するオキシダーゼ(特開平3-155780号公報)、ギベレラ属菌の生産するオキシダーゼ(特開平7-289253号公報)、フサリウム属菌の生産するオキシダーゼ(特開平7-289253号公報、特開平8-154672号公報)、ペニシリウム属菌の生産するオキシダーゼ(特開平8-336386号公報)さらには、ケトアミノオキシダーゼ(特開平5-192193号公報)などを挙げることができる。

【0019】既知のフルクトシルアミノ酸オキシダーゼなどを改変することにより、本発明のオキシダーゼを得るためには、上記既知のフルクトシルアミノ酸オキシダーゼなどの生産能を有する微生物に、紫外線、X線、放射線などを照射したり、もしくは、エチルメタンサルフォネート、N-メチル-N'-ニトロ-N-ニトロソグアニジン、亜硝酸などの変異誘発剤を接触させることにより、変異処理をする。得られた変異処理微生物から、本発明のオキシダーゼを生産する微生物を選抜する。

【0020】しかし、一般的には、上記既知のフルクトシルアミノ酸オキシダーゼなどの遺伝子(以下、野生型遺伝子と言う)に変異を導入することにより、本発明のオキシダーゼを得ることができる。変異を導入するために用いられる野生型遺伝子は、例えば、上記フルクトシルアミノ酸オキシダーゼ及び類似のオキシダーゼ等の野生型遺伝子で、変異を導入することにより、本発明のオキシダーゼを得ることができる野生型遺伝子であれば、

如何なる遺伝子でも用いることができる。

【0021】上記野生型遺伝子は、フルクトシルアミノ酸オキシダーゼ、又は類似のオキシダーゼなどを生産する能力を有する生物、好ましくは微生物由来の天然の遺伝子をクローニングすることにより得られる。クローニングの方法としては、先ず上記オキシダーゼを生産する生物から、通常用いられている、例えば、Current Protocols in Molecular Biology (WILEY Interscience, 1989) 記載の方法等により、染色体DNA又はmRNAを抽出する。さらにmRNAを鋳型としてcDNAを合成することができる。このようにして得られた染色体DNA又はcDNAのライブラリーを作製する。次いで、上記オキシダーゼなどのアミノ酸配列に基づき、適当なプローブDNAを合成して、これを用いてDNA又はcDNAのライブラリーからスクリーニングする方法、あるいは、該ペプチドのアミノ酸配列に基づき、適当なプライマーDNAを作製して、5' RACE法や3' RACE法などの適当なポリメラーゼ連鎖反応(PCR法)により、目的の遺伝子断片を含むDNAを増幅させ、これらを連結させて全長の野生型遺伝子を含むDNAを取得する方法等が挙げられる。さらに、一例として、一般に入手可能な遺伝子源として、コリネバクテリウム属菌由来の本発明の野生型遺伝子をコードするプラスミドDNAを保持する大腸菌DH5 $\alpha$  (pFA5) (FERM BP-6182) から、常法に従って単離する方法を挙げることができる。

【0022】野生型遺伝子に変異を導入する方法としては、野生型遺伝子と変異剤、例えば、ニトロソグアニジン等のアルキル化剤、アクリジン色素、ヒドロキシルアミン、亜硝酸、亜硫酸、5-ブロモウラシル、ベンゾピレンなどを接触させる方法を挙げることができる。この他、紫外線照射法、トランスポゾン、カセット式変異法、キメラ遺伝子作製法、PCR法、DNAシャフリング法などを用いた変異導入方法を広く用いることができる。また、変異を導入するための野生型遺伝子は、適当なベクターDNAに挿入されたもの、即ち組換え体DNAであってもよく、その場合、変異処理後の組換え体DNAをエタノール沈殿などで精製する。得られた変異型遺伝子は、組換え体DNAを用いた宿主細胞の形質転換あるいは形質導入などによって発現させることができる。変異型遺伝子を保持する多数の菌株より、本発明のオキシダーゼを生産する菌株を選抜する。

【0023】目的の微生物や菌株を選抜する方法としては、基質として $\alpha$ -糖化ペプチド、好ましくは、 $\alpha$ -糖化ジペプチド、 $\alpha$ -糖化トリペプチド、 $\alpha$ -糖化テトラペプチドなどの短鎖の $\alpha$ -ペプチドが挙げられる。一例として、 $\alpha$ -糖化ジペプチドとして、フルクトシルVal-Hisなどを使用する方法などが挙げられる。この基質を含む反応液に、検定するための微生物または菌株の菌体より、破碎処理や溶菌処理により得られた又はそ

れらの遠心上清より得られた酵素抽出液を添加して反応させ、生成する過酸化水素を、後述する通常用いられている過酸化水素発色基質により発色させて、本発明のオキシダーゼを生産する微生物または菌株を選抜する。酵素抽出液は、そのまま用いても良いが、場合によっては濃縮又は希釈して用いることもできる。また酵素反応により減少する酸素量を酸素電極により測定することもできる。選抜には、試験管内で酵素反応を行なっても良いが、96穴マイクロプレートウェル内で反応させる方法、酵素抽出液を吸着させた膜に反応試薬を塗布又は浸透させることで反応させる方法や酵素抽出液を吸着させた膜に反応試薬を塗布した膜を重ね合わせて反応させる方法などを適宜採用することもできるし、複数の菌株を混ぜて、数段階の選抜を行なうことで、効率良く、簡便に行なうこともできる。

【0024】このようにして、既知のコリネバクテリウム属菌の生産するフルクトシルアミノ酸オキシダーゼ(特公平5-33997号公報、特公平6-65300号公報)を改変して得られた本発明のオキシダーゼを生産する菌株として、具体的に、大腸菌(E. coli) DH5 $\alpha$  (pFP1) を挙げることができる。大腸菌(E. coli) DH5 $\alpha$  (pFP1) は、工業技術院生命工学工業技術研究所にFERM P-17576として寄託されている。

【0025】本発明のオキシダーゼは、該酵素を含む動物、植物などの組織や該酵素を生産する微生物より、通常用いられている抽出方法などをもちいて得られる。例えば、本発明のオキシダーゼを生産する微生物を用いて、本発明のオキシダーゼを製造するには、以下のようにして行なうことができる。上記微生物を培養するには、通常の固体培養法で培養してもよいが、可能なかぎり液体培養法を採用して培養するのが好ましい。培養に用いる培地は、炭素源、窒素源、無機物、その他の栄養素を適宜含有するものであればよく、また合成培地、天然培地の何れでもよく、目的の酵素を効率よく製造することのできる培地であれば、如何なる培地でもよい。炭素源としては、同化可能な炭素化合物であればよく、例えばグルコース、デンプン加水分解物、グリセリン、フラクトース、糖蜜などが挙げられる。窒素源としては、利用可能な窒素化合物であればよく、例えば酵母エキス、ペプトン、肉エキス、コーンスチープリカー、大豆粉、マルツエキス、アミノ酸、硫酸アンモニウム、硝酸アンモニウムなどが挙げられる。無機物としては、例えば、食塩、塩化カリウム、硫酸マグネシウム、塩化マンガ、硫酸第1鉄、リン酸第1カリウム、リン酸第2カリウム、炭酸ナトリウム、塩化カルシウムなどの種々の塩が挙げられる。その他、必要に応じてビタミン類、消泡剤などを添加してもよい。また本発明のオキシダーゼが作用する基質やそれに類似の物質、例えば、糖化ペプチド類、フルクトシルアミノ酸、糖化蛋白部分分解物、

糖化ヘモグロビン、糖化アルブミン、糖と共に加温する処理などにより人工的に糖化した糖化ペプチド、糖化蛋白質なども添加することにより目的の酵素の製造量を向上せしめることができる。これらの栄養源や添加する物質はそれぞれ単独で用いてもよいが、組み合わせて用いてもよい。培養条件は、培養する微生物により異なる。例えば、培地の初発 pH は、pH 5~10 に調整し、培養温度は、20~40℃、培養時間は、10~50 時間、好ましくは 15~25 時間、通気攪拌深部培養、振盪培養、静地培養などにより実施する。

【0026】培養終了後、該培養物から、本発明のオキシダーゼを採取するには、通常の酵素の採取手段を用いることができる。上記酵素が菌体内に存在する場合には、培養物から、例えば濾過、遠心分離などの操作により菌体を分離し、この菌体から酵素を採取するのが好ましい。例えば、超音波破砕機、フレンチプレス、ダイノミルなどの、通常の破壊手段を用いて菌体を破壊する方法、リゾチームなどの細胞壁溶解酵素を用いて菌体細胞壁を溶解する方法、トリトン X-100 などの界面活性剤を用いて菌体から酵素を抽出する方法などを単独又は組み合わせて採用することができる。次いで、濾過又は遠心分離などにより不溶物を取りのぞき、酵素抽出液を得る。得られた抽出液から本発明のオキシダーゼを、必要に応じて単離、精製するには、必要により、ストレプトマイシン硫酸塩、プロタミン硫酸塩、硫酸マンガンなどにより核酸を除去したのち、これに硫酸アンモニウム、アルコール、アセトンなどを添加して分画し、沈殿物を採取し、粗酵素を得る。さらに精製酵素標品を得るには、例えば、セファデックス、ウルトラゲルもしくはバイオゲルなどを用いるゲル濾過法、イオン交換体、ヒドロキシアパタイトなどを用いる吸着溶出法、アフィニティクロマト法、分子ふるい膜もしくは中空糸膜などを用いる分画法などを適宜選択し、またこれらを組み合わせて実施することにより、目的の精製度の酵素標品を得ることができる。上記酵素が菌体外に存在する場合には、常法により、前述の菌体分離操作の後、培養液を回収・濃縮し、上記各種精製方法に供すればよい。

【0027】本発明のオキシダーゼの力価は、例えば、下記の方法で測定することができるが、他の方法でも測定可能であり、この測定方法に限るものではない。

#### (1) 試薬の調製

試薬 1 (R1) : 1.0 kU のパーオキシダーゼ (TYPE III, 東洋紡社製)、100 mg の 4-アミノアンチピリン (東京化成社製) を 0.1 M のリン酸カリウム緩衝液 (pH 8.0) に溶解し、1 L に定容する。

試薬 2 (R2) : 500 mg の TOOS (N-エチル-N-(2-ヒドロキシ-3-スルホプロピル)-m-トリジン、同仁化学社製) をイオン交換水に溶解し、100 mL に定容する。

試薬 3 (R3) : フルクトシル Val-His (MW 4

16、製造方法は後述) 1.25 g をイオン交換水に溶解し、10 mL に定容する。

#### (2) 測定

2. 7 mL の R1 に、100  $\mu$ L の R2 を加え、さらに、100  $\mu$ L の本発明のオキシダーゼを含む酵素液を加えて混和し、37℃で、5 分間予備加温する。その後、100  $\mu$ L の R3 を加えてよく混ぜたのち、分光光度計 (U-2000A、日立社製) を用い、37℃、5 分間の 555 nm における吸光度の変化を測定する。なお、対照液は、100  $\mu$ L の R3 の代わりに、100  $\mu$ L のイオン交換水を加える以外は前記と同様に操作する。あらかじめ調製した過酸化水素の標準溶液を用いて、その生成する色素量 (吸光度) との関係より得られたグラフから、吸光度の変化に相当する過酸化水素量を求め、この数値を酵素液中の活性単位とする。1 分間に 1  $\mu$ mol の過酸化水素を生成する酵素量を 1 U とする。

【0028】このようにして得られた本発明のオキシダーゼは、糖化ペプチドに特異的に作用し、過酸化水素を生成する性質を有することから、生体中や食品等に含まれる糖化ペプチドを酵素的に測定することができるだけでなく、生体中の糖化蛋白質をプロテアーゼで処理して得られる、遊離した糖化ペプチドを酵素的に測定することができ、本発明の糖化蛋白質の測定用試薬に好適に用いられる。

【0029】プロテアーゼ処理により遊離した糖化ペプチドに上記本発明のオキシダーゼを作用させる。用いる本発明のオキシダーゼは処理液中に含まれる糖化ペプチドの量にもよるが、例えば、終濃度が、0.1~50 U/mL、好ましくは 1~10 U/mL となるように添加すればよい。作用させるときの pH は、例えば、pH 3~11、特に好ましくは pH 5~9 であり、本発明のオキシダーゼの至適 pH を考慮し、本発明の測定に適した pH となるように、緩衝剤を用いて調整するのが好ましいが、作用可能な pH であればこれに限定されない。pH の調製方法は特に限定されないが、緩衝剤としては、例えば、N-[トリス (ヒドロキシメチル) メチル] グリシン、リン酸塩、酢酸塩、炭酸塩、トリス (ヒドロキシメチル) -アミノメタン、硼酸塩、クエン酸塩、ジメチルグルタミン酸塩、トリシン、HEPES などが挙げられる。また、必要により適宜、プロテアーゼ処理後の処理液の pH を、緩衝剤を用いて上記 pH に調整してもよい。作用時間は、例えば、1~120 分、好ましくは 1~30 分であり、基質となる糖化ペプチドの量にもよるが、本発明のオキシダーゼが、それらのペプチドに作用するのに十分な時間であればよい。作用温度は、例えば、20~45℃であり、通常の酵素反応に用いられる温度を適宜選択することができる。

【0030】本発明では、遊離した糖化ペプチドに本発明のオキシダーゼを作用させ、その作用による生成物ま

たは消費物を測定することにより、糖化ペプチドを測定する。本発明のオキシダーゼの作用により、糖化ペプチドから生成する物質としては、例えば、ペプチド、過酸化水素および糖オゾンなどが挙げられる。一方、消費される物質としては、酸素分子などが挙げられる。これらの生成物や消費物をそれぞれの測定方法を用いて測定する。例えば、酸素分子は酸素電極を用いる電気的方法、ペプチドは逆相HPLCを用いる分離、測定方法などが挙げられるが、好ましくは、短時間で簡単に測定できる方法として、過酸化水素を測定する方法が挙げられる。

【0031】本発明のオキシダーゼの作用により生成した過酸化水素は、如何なる方法により測定してもよいが、例えば、酸素電極を用いる電気的方法、好ましくは、パーオキシダーゼおよび適当な発色基質を用いる酵素的方法などが挙げられる。例えば、本発明では、酵素的方法を用いて、短時間に、簡単な操作で測定を行なうことが好ましい。本発明の酵素的方法により過酸化水素を測定するための試薬としては、例えば、緩衝剤(pH 4~10が好ましい) 5~500mM、好ましくは50~100mM、発色基質として4-アミノアンチピリン 0.01~50mM、好ましくは0.1~20mM、パーオキシダーゼ0.1~50U/mL、好ましくは1~20U/mLなどの組成を含む試薬を挙げることができる。本発明に用いられる緩衝剤としては、例えば、N-[トリス(ヒドロキシメチル)メチル]グリシン、リン酸塩、酢酸塩、炭酸塩、トリス(ヒドロキシメチル)-アミノメタン、硼酸塩、クエン酸塩、ジメチルグルタミン酸塩、トリシン、HEPESなどが挙げられる。発色基質としては、4-アミノアンチピリンの他に、例えば、ADOS(N-エチル-N-(2-ヒドロキシ-3-スルホプロピル)-m-アニジジン)、ALOS(N-エチル-N-(2-ヒドロキシ-3-スルホプロピル)アニリン)、10-(カルボキシメチルアミノカルボニル)-3,7-ビス(ジメチルアミノ)-フェノシアジン(DA-67)、N-(カルボキシメチルアミノカルボニル)-4,4'-ビス(ジメチルアミノ)-ジフェニルアミン(DA-64)などが挙げられる。さらに必要に応じて、本発明の目的を損なわない範囲で、種々の添加物、例えば、溶解補助剤、安定化剤などとして、界面活性剤(トリトンX-100、ブリッジ35、ツイーン80、コール酸塩など)、還元剤(ジチオスレイトール、メルカプトエタノール、L-システインなど)、牛血清アルブミン、糖類(グリセリン、乳糖、シュクロースなど)などを適宜添加してもよい。

【0032】この過酸化水素の測定を行なうとき、一般に、オキシダーゼの作用により過酸化水素を生成する工程を同時に行なうことが好ましいため、本発明では、前述の過酸化水素を測定するための試薬に、本発明のオキシダーゼを例えば、0.1~50U/mL、好ましくは1~10U/mL添加することが好ましい。これらの測

定用試薬は、乾燥物又は溶解した状態で用いてもよいし、薄膜上の担体、例えば、シート含浸性の紙などに含浸させて用いてもよい。また本発明の測定用試薬に用いられる酵素類は、常法により固定化させて反復使用することもできる。測定温度は、例えば、20~45℃であり、通常の酵素反応に用いられる温度を適宜選択することができる。測定に要する時間は、種々の測定条件により適宜選択できるが、例えば、0.1~60分、特に、本発明では1~10分が好ましい。上記測定試薬の発色の程度(吸光度変化量)を分光光度計により測定し、標準の吸光度と比較して、試料中に含まれる糖化ペプチドや糖化蛋白質を測定することができる。測定には、通常の自動分析装置を用いることもできる。

【0033】本発明の試料中の糖化蛋白質を測定するための測定用試薬キットは、糖化蛋白質より糖化ペプチドを遊離するために用いるプロテアーゼ、本発明のオキシダーゼ及び過酸化水素を測定するための試薬などの成分を含む。それぞれの成分の具体的な組成は、前記した組成を用いることができる。上記成分は、それぞれ別々に保存して、使用してもよいし、本発明のオキシダーゼと過酸化水素を測定するための試薬は合わせて保存、使用してもよい。また、本発明では上記試薬キットを用いて糖化蛋白質を測定するとき、例えば、糖化ペプチドを遊離させる工程と遊離したその糖化ペプチドを測定する工程とを別々に2段階で測定することもできるし、それらの成分を合わせて、上記工程を連続的に1段階で測定することもできる。

【0034】次に、遊離した糖化ペプチドをHPLCを用いて測定する方法について述べる。遊離した糖化ペプチドを含むプロテアーゼ処理液をそのまま、もしくは必要により、処理液を遠心濾過や膜濾過した濾過液を、適宜、濃縮・希釈した後、HPLC測定に用いる。本発明に用いるHPLCは、上記糖化ペプチドを測定することが可能なHPLCであれば、如何なるHPLCでも用いることができる。例えば、用いる逆相HPLCカラムとして、CAPCEL-PAK C-18(資生堂製)、TSKgel ODS80Ts(東ソー社製)、Shodex RSpak RP18-415(昭和電工社製)、イオン交換HPLCカラムとして、TSKgel SP-2SW、TSKgel CM-2SW(東ソー社製)等が挙げられる。これらのカラムにプロテアーゼ処理液を吸着させた後、溶離液を用いて、目的とする糖化ペプチドを溶出する。溶離液としては、本発明の測定に適した溶離液であれば、如何なる溶離液でも良いが、例えば、逆相カラムではトリフルオロ酢酸を含むアセトニトリルと水との混合液、リン酸緩衝液とアセトニトリルとの混合液、アンモニア水溶液とアセトニトリルとの混合液等、イオン交換カラムでは、例えば、リン酸緩衝液とNaCl溶液との混合液、酢酸緩衝液とアセトニトリルとの混合液等が用いられる。これらの溶離液を



用いて、ステップワイズに溶離しても良いし、グラジエントに溶離しても良い。好ましい溶離液として、例えば、0.1%TFA（トリフルオロ酢酸）／水-0.1%TFA／30%アセトニトリルのグラジエント溶離液などを挙げることができる。本発明では、用いるカラム、溶離液、溶離条件（溶離方法、溶離液の流速、温度等）等を適宜、組合せ、目的の $\alpha$ -糖化ペプチドの溶出ピークが、できる限り他の成分のピークと良好に分離する条件に設定することが好ましい。

【0035】溶離液により溶出された糖化ペプチドを検出する方法としては、糖化ペプチドを検出することのできる検出方法であれば如何なる方法でも用いることができるが、例えば、波長210nm、215nm等における吸光度を検出する方法、各検出ピークを分取した後、マスマススペクトロメトリー分析に供して目的分子量のピークを決定する方法、薄層クロマトグラフィーに供する方法、あるいは、経時的に分取した溶出画分をニンヒドリン法や糖発色法で比色定量する方法等が用いられる。一例として、例えば、吸光度を検出する方法を用いる場合、モニターにより検出された糖化ペプチドの溶出ピーク面積を算出して、標準物質の溶出ピーク面積と比較して、その糖化ペプチドの量及び糖化蛋白質を測定することができる。

#### 【0036】

【実施例】以下、参考例および実施例により、本発明をさらに具体的に説明する。但し、本発明の技術的範囲は、それらの例により、何ら限定されるものではない。

#### 【0037】参考例（糖化ジペプチドの製造）

本発明において使用する $\alpha$ -糖化ジペプチドは、以下に示す方法で製造した。市販のジペプチド（バリンヒスチジン（Val-His）、スイス、BACHEM社製）7.0g（27.6mmol）を14mLの水に溶解し、酢酸5.8mLを加え、約50℃で溶解、澄清化させた。次いで、エタノール120mLを添加し混合後、グルコース14g（77.8mmol）を添加し、よく混合した。その後、密閉容器内で80℃、6時間、ときどき攪拌を行ないつつ加温処理を行なった。反応液は経時的に褐変した。反応溶液を経時的に分取し、適宜希釈後、逆相高速液体クロマトグラフィー分析、薄層クロマトグラフィー分析、あるいはマスマススペクトロメトリー分析に供することにより、目的の糖化ジペプチドの生成を検定した。通常、6～10時間の加温処理により、良好な収率で糖化ジペプチドを得ることができる。次いで、反応溶液を回収し、ロータリーエバポレーターを用いて、15～30倍に濃縮した。濃縮物を、99.5%エタノールで平衡化したシリカゲルカラム（容量2000mL）に吸着させ、カラムの2倍量の99.5%エタノールで洗浄し、未反応のグルコースなどの夾雑成分を除去した後、3倍量の95%エタノール、3倍量の90%エタノール、3倍量の85%エタノール、3倍量の80

%エタノールで順次溶出を行なった。各溶出画分を薄層クロマトグラフィー分析、逆相高速液体クロマトグラフィー分析などで分析し、目的のフルクトシルVal-Hisを含む95～90%エタノール溶出画分を回収した。ロータリーエバポレーターを用いて回収物を濃縮乾固させ、約3gの部分精製物を得た。マスマススペクトロメトリー分析の結果、この精製物の分子量はMW416であり、フルクトシルVal-Hisの分子量と一致し、また、核磁気共鳴スペクトル分析により、その構造を確認した。この部分精製物を、常法により、イオン交換樹脂を用いて吸脱着し、精製度を高め、以後の実験に用いた。更にトリペプチド、テトラペプチド、ヘキサペプチドを用いて、上記と同様の方法で、それぞれ糖化トリペプチド、糖化テトラペプチド、糖化ヘキサペプチドの部分精製物を得た。

#### 【0038】実施例1（糖化ヘキサペプチドより糖化ジペプチドの遊離）

糖化ヘモグロビン（HbA1c）を、エンドプロテイナーゼGlucで処理することにより、糖化ヘモグロビン（HbA1c）より、その $\beta$ サブユニット由来の $\alpha$ -糖化ヘキサペプチド（フルクトシルVal-His-Leu-Thr-Pro-Glu）が遊離する（Clin. Chem., 43, 1994-1951 (1997)）。そこで、この $\alpha$ -糖化ヘキサペプチドと同一物質である、ペプチド研究所社製のフルクトシルVal-His-Leu-Thr-Pro-Gluを用いて、以下の実験を行なった。

【0039】上記 $\alpha$ -糖化ヘキサペプチド（ペプチド研究所社製）を水に溶解し、5mM溶液とした。この溶液0.1mLに、下記のプロテアーゼ溶液（20mg/mL）0.01mLおよび緩衝液（0.1M）0.09mLを添加、混合してプロテアーゼ処理を行なった。

(a) カルボキシペプチダーゼY（オリエンタル酵母社製）、リン酸緩衝液（pH6.5）。

(b) AOプロテアーゼ（（株）盛進、販売）、クエン酸-リン酸2ナトリウム緩衝液（pH6.0）。

(c) ペプチダーゼ（（株）盛進、販売）、クエン酸-リン酸2ナトリウム緩衝液（pH6.0）。

(d) モルシン（（株）盛進、販売）、クエン酸-リン酸2ナトリウム緩衝液（pH3.0）。

【0040】上記混合物を37℃、60分間反応処理した。その後、処理液をそれぞれ、適宜濃縮・希釈し、HPLCにて測定した。HPLC（逆相高速液体クロマトグラフィー）として、CAPCEL-PAK C-18（資生堂社製）を用いた。溶離液として、0.1%TFA（トリフルオロ酢酸）／水-0.1%TFA／30%アセトニトリルを用い、グラジエントで溶離した。標準物質として、 $\alpha$ -糖化ジペプチド（フルクトシルVal-His）を用いた。更に溶出した糖化ペプチドを薄層クロマトグラフィー（シリカプレート、メルク社製）を用い、展開溶媒はn-ブタノール：酢酸：水=2：1：1

1、スポット検出はニンヒドリン及びエタノール-硫酸)で確認した。その結果、(a)、(b)、(c)、

(d)の何れにおいても、プロテアーゼ処理液中に $\alpha$ -糖化ジペプチド(フルクトシルVal-His)が遊離していることが解った。さらに、各処理液を、アミノ酸分析(日立アミノ酸分析計 L-8800)およびマス

スペクトロメトリー分析(日立質量分析計 Model M-80B)に供した。遊離したアミノ酸残基の同定とその分子量の測定結果から、何れのプロテアーゼ処理液においても $\alpha$ -糖化ヘキサペプチド(フルクトシルVal-His-Leu-Thr-Pro-Glu)が、カルボキシ末端から順に、及び/または内部切断的に、切断され、より短鎖の $\alpha$ -糖化ペプチドに分解されていることを確認した。(a)の場合、カルボキシ末端から、Glu、Pro、Thr及びLeu残基の遊離が確認される一方、His残基の遊離が確認されないことにより、フルクトシルVal-Hisまで短鎖化されたことが確認された。さらに、処理液のマススペクトロメトリー分析では、処理後に確認された糖化ペプチドの大部分がフルクトシルVal-Hisであり、わずかに、フルクトシルVal-His-Leu及びフルクトシルVal-His-Leu-Thrに相当する分子量のシグナルが認められたにすぎなかった。(b)及び(c)では、フルクトシルVal-Hisと少量のフルクトシルVal-His-Leuのシグナルを認めた。また、(d)では、フルクトシルVal-Hisのシグナルのみを認めた。

#### 【0041】実施例2 (糖化蛋白質より糖化ジペプチドの遊離)

糖化ヘモグロビン(HbA1c)コントロール(国際試薬社製)に蒸留水を加え、8g/dL(HbA1c含有率約10%)の溶液を調製した。この溶液0.05mLにアスペルギルス属由来プロテアーゼ(モルシン、20mg/mL)0.01mL、及び緩衝液(0.1M、クエン酸-リン酸2ナトリウム緩衝液、pH3.0)0.04mLを添加、混合した。混合液を37℃、180分間プロテアーゼ処理をした後、処理液をマイクロコン3(分画分子量3000、グレースジャパン社製)で遠心濾過し、濾過液を希釈した後、実施例1に記載のHPLCにて測定した。フルクトシルVal-Hisの遊離を確認し、その溶出ピーク面積から、糖化ジペプチドを測定することができた。この測定値より、糖化蛋白質を測定することができた。

#### 【0042】実施例3 (改変された本発明オキシダーゼの取得)

##### (1) 鋳型DNAの調製

コリネバクテリウム属菌由来のフルクトシルアミノ酸オキシダーゼ遺伝子をコードするプラスミドを保持する大腸菌DH5 $\alpha$ (pFA5)(FERM BP-6182)をLB-amp培地(1%バクトトリプトン、0.

5%バクトイースト・エキストラクト、0.5%塩化ナトリウム、50 $\mu$ g/mLアンピシリン、pH7.0)100mLに接種して、30℃で24時間振盪培養し、培養物を得た。この培養物より、Molecular Cloning(2nd. Edition, 1989)に記載の方法に従い、pFA5プラスミドDNA1.5mgを得た。

##### (2) 変異の導入

pFA5プラスミドDNA30 $\mu$ gを100 $\mu$ Lのヒドロキシルアミン溶液(0.8M塩酸ヒドロキシルアミン/0.1Mリン酸緩衝液、pH6.0/1mMEDTA)に溶解し、65℃で2時間変異処理した後、常法によりエタノール沈殿を行い、沈殿物を回収した。この沈殿物をTE緩衝液(10mMトリス-塩酸緩衝液、pH7.5/1M EDTA)に溶解し、D.M.Morrisonの方法(Method in Enzymology, 68, 326-331, 1979)により、大腸菌DH5 $\alpha$ 株を形質転換し、LB-amp寒天培地(1%バクトトリプトン、0.5%バクトイースト・エキストラクト、0.5%塩化ナトリウム、50 $\mu$ g/mLアンピシリン、1.5%(w/v)アガロース、pH7.0)に接種し、30℃で24時間培養した。

##### (3) 生産菌の選抜

18時間培養後出現してきたコロニー、約50000株を30mg/mLのLysozyme溶液に浸したHybond-Cに転写し、一方、50mMフルクトシルVal-His、0.5mg/mLパーオキシダーゼ、1.0mg/mL4-アミノアンチピリン、50mg/mL TOOS、100mMリン酸カリウム緩衝液(pH8.0)に浸したHybond-Cを用意し、両者を菌体表が内側になるように重ね合わせ、37℃で30分～1時間程度反応させた。ここで発色の認められた3株を選抜し、LB-amp培地10mLに接種して、30℃で24時間振盪培養した後、培養液を超音波処理にて破碎し、遠心分離後、その上清について、前記した方法で、糖化ペプチドオキシダーゼ活性を測定したところ、1株に活性を認めた。この株を大腸菌DH5 $\alpha$ (pFP1)とした。

##### (4) 酵素の製造

選抜された本発明の糖化ペプチドオキシダーゼ生産能を有する大腸菌DH5 $\alpha$ (pFP1)をLB-amp培地10Lに植菌し、ジャーファーメンターを用いて、通気量1L/min、攪拌速度600rpmの条件で、30℃、20時間攪拌培養した。得られた培養液20LをMW50,000の限外濃縮膜(旭化成社製)で5Lまで濃縮し、1Mリン酸カリウム緩衝液(pH8.0)を加えた。その後、ダイノミルにより菌体を破碎した。破碎液を10,000rpmで15分間遠心分離し、得られた上澄み液を粗酵素液とし、以下の方法で精製した。

【0043】粗酵素液に、0.15Mとなるように塩化カリウムを加え、0.15M塩化カリウムを含有する50mMリン酸カリウム緩衝液(pH8.0)で平衡化し

た、DEAE-セファセルカラム 2Lに吸着させた。2Lの同じ緩衝液で洗浄した後、塩化カリウム濃度0.15M~0.50Mの直線勾配のリン酸カリウム緩衝液(pH8.0)で溶出させた。得られた溶出液について、前記本発明のオキシダーゼの力価の測定方法に基づき活性を測定した後、活性画分を集め、得られた酵素液をMW6,000の限外濃縮膜(旭化成社製)で濃縮し、16%硫酸アンモニウムを含有する50mMリン酸カリウム緩衝液(pH8.0)で透析した。次に、16%硫酸アンモニウムを含有する50mMリン酸カリウム緩衝液(pH8.0)で平衡化したブチルトヨパールカ

#### 試薬A(発色試薬)

4-アミノアンチピリン(東京化成社製)

TOOS

パーオキシダーゼ(東洋紡社製)

リン酸カリウム緩衝液(pH8.0)

#### 試薬B(オキシダーゼ試薬)

本発明のオキシダーゼ

リン酸カリウム緩衝液(pH8.0)

参考例に記載の方法で得られた $\alpha$ -糖化ジペプチド、フルクトシルVal-Hisを用いて、1.0mmol/L溶液を調製した。この溶液を希釈して、種々の濃度(25、50、75、及び100 $\mu$ mol/L)の糖化ジペプチド含有試料を調製した。この含有試料、各0.3mLに、試薬A2.1mLを添加し、37℃で5分間、保温した。それぞれの保温液に試薬B0.6mLを添加し、37℃で10分間反応させた。555nmにおける吸光度を測定し、反応10分後の吸光度の増加量( $\Delta$ OD)を求めた。各種濃度の $\alpha$ -糖化ジペプチドの測定結果の一例を図1に示す。図1から、 $\Delta$ ODと $\alpha$ -糖化ジペプチドの濃度との間には直線的な相関のあることが示された。試料中の $\alpha$ -糖化ジペプチドを短時間でかつ精度よく測定できることがわかった。一方、試薬B(オキシダーゼ試薬)の本発明のオキシダーゼの代わりに、既知のコリネバクテリウム属菌の生産するフルクト

#### 試薬C(プロテアーゼ試薬)

モルシン(株)盛進 販売)

塩化カリウム-塩酸緩衝液(pH3.0)

ヒト溶血液より常法(遠心分離、濃縮透析、及びイオン交換高速液体クロマトグラフィー法等の組合せ)により分取した、非糖化ヘモグロビン、及び糖化ヘモグロビン(HbA1c)画分を適当な比率で混合し、全ヘモグロビンに対するHbA1c含有率(HbA1c値)が0~50%の数種の試料を調製した。この試料100 $\mu$ Lに、試薬C(プロテアーゼ試薬)100 $\mu$ Lを添加し、37℃、1時間プロテアーゼ処理した後、この処理液を煮沸し、プロテアーゼ反応を停止させた。次いで、この処理液に0.5M NaOHを添加して、pH7とした後、遠心分離(12000rpm、5分間)し、上清を分取した。この上清0.3mLに、試薬A(発色試薬)

ラムに、吸着させ、同じ緩衝液で洗浄した後、硫酸アンモニウム濃度16%~0%の直線勾配の50mMリン酸カリウム緩衝液(pH8.0)で溶出させ、活性画分を回収した。続いて、この酵素液をMW6,000の限外濃縮膜(旭化成社製)で濃縮し、50mMリン酸カリウム緩衝液(pH8.0)で透析し、目的の酵素液を得た。

【0044】実施例4 (オキシダーゼを用いる $\alpha$ -糖化ジペプチドの測定)

糖化ジペプチドの測定に用いる、以下の試薬を調製した。

0.2mM

0.2mM

14.3U/mL

0.1M

4U/mL

0.02M

シルアミノ酸オキシダーゼ(特公平5-33997号公報、特公平6-65300号公報)4U/mLを用いて上記と同様に測定を行なったが、何れの試料についても吸光度の増加量( $\Delta$ OD)を測定することはできなかった。これらのことから、既知のフルクトシルアミノ酸オキシダーゼを改変することにより、新たに、糖化ペプチドに作用する活性を有する本発明のオキシダーゼの得られることが解った。

【0045】実施例5 (オキシダーゼを用いる糖化蛋白質の測定)

本発明のオキシダーゼを用いる糖化蛋白質の測定に用いる、以下の試薬を調製した。

#### 試薬A(発色試薬)

実施例4に記載の通り。

#### 試薬B(オキシダーゼ試薬)

実施例4に記載の通り。

20mg/mL

100mM

2.1mL、及び試薬B(オキシダーゼ試薬)0.6mLを添加、混合し、37℃、30分間反応させた。反応開始前、及び反応終了後の555nmにおける吸光度を測定し、該吸光度の増加量( $\Delta$ OD)の値を求めた。HbA1c値の異なる数種の試料について、測定した結果の一例をを図2に示す。この結果から、 $\Delta$ ODと初発試料中のHbA1c量との間には、直線的な相関が認められた。これにより、試料中の糖化ヘモグロビンを簡便に、迅速かつ精度良く測定できることがわかった。

【0046】

【発明の効果】本発明の測定方法は、糖化蛋白質、例えば糖化ヘモグロビンなどを、短時間で、簡単な操作で、

精度よく測定することができ、糖尿病の症状の診断や症状管理に有効に用いられる。

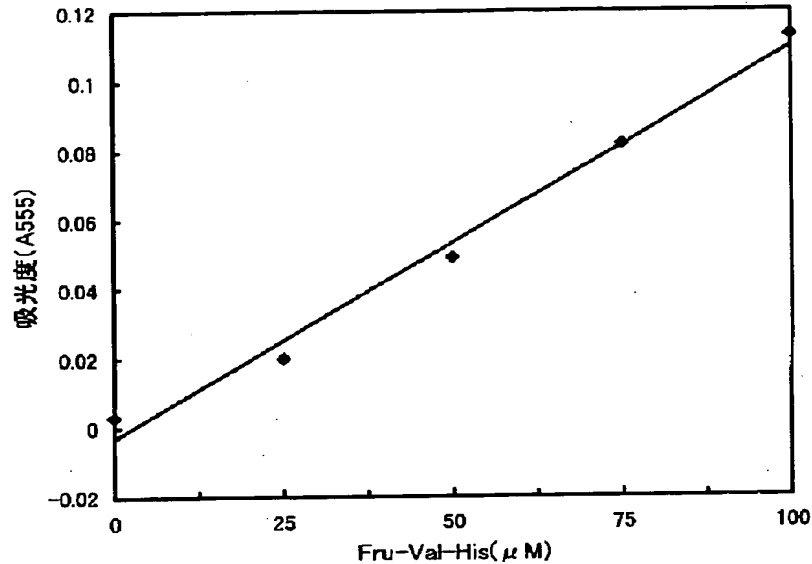
【図面の簡単な説明】

【図1】  $\alpha$ -糖化ジペプチド（フルクトシルバリルヒ

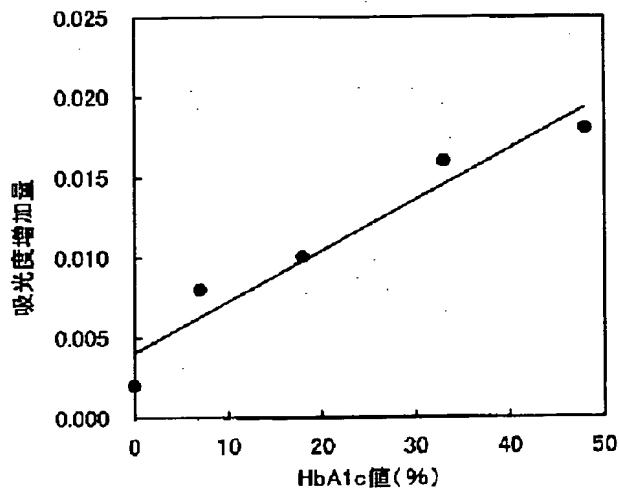
スチジン）の測定結果を示す図。

【図2】 糖化ヘモグロビン（HbA1c）の測定結果を示す図。

【図1】



【図2】



【手続補正書】

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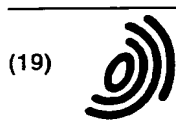
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(54) **METHOD OF ASSAYING GLYCOPROTEIN**

(57) Based on a principle that is different to that of a conventional enzymatic method, the present invention provides a novel method for assaying a glycosylated protein by a simple procedure, within a short period of time, and with high accuracy, and a reagent kit for assaying used in the method. The method for assaying a glycosylated protein in a sample is realized by treating a glycosylated protein-

containing sample with protease to liberate a glycosylated peptide, preferably an  $\alpha$ -glycosylated peptide, particularly preferably an  $\alpha$ -glycosylated dipeptide, from a glycosylated protein, allowing an oxidase to react with the liberated glycosylated peptide, and determining the produced hydrogen peroxide.

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## Description

## TECHNICAL FIELD

[0001] The present invention relates to a method for assaying a glyated protein in a sample and a reagent kit for assaying, which is used in the assay method.

## BACKGROUND ART

[0002] A glyated protein is a protein that has been glycosylated in a nonenzymatic manner and produced as a result of nonenzymatic covalent binding between an aldehyde group on a saccharide, i.e., an aldose (a monosaccharide potentially having an aldehyde group or a derivative thereof), and an amino group on the protein. These glyated proteins are also referred to as so-called "Amadori compounds" since they are formed through Amadori rearrangement of a Schiff's base produced as a reaction intermediate.

[0003] Glyated protein is contained in biological samples including hair and body fluid such as blood in an organism. The concentration of glyated protein that is present in blood strongly depends on the concentration of saccharides such as glucose dissolved in blood serum. With a diabetic condition, production of glyated protein is accelerated and the concentration of glyated hemoglobin contained in erythrocyte or the concentration of glyated albumin in blood serum reflects the average blood glucose level of the specific past period. Thus, the assay of those glyated proteins is important in diagnosis or control of diabetic symptoms.

[0004] Conventional methods known as methods for quantitatively analyzing a glyated protein include a method utilizing high performance liquid chromatography (Chromatogr. sci., 10, 659 (1979)), a method utilizing a column in which a solid prepared by binding a boric acid is packed (Clin. Chem., 28, 2088-2094 (1982)), a method utilizing electrophoresis (Clin. Chem., 26, 1598-1602 (1980)), a method utilizing an antigen-antibody reaction (JJCLA, 18, 620 (1993)), a method for performing colorimetric measurement of reducibility using tetrazolium salt (Clin. Chim. Acta, 127, 87-95 (1982)), and a method for performing colorimetric measurement after oxidation with thiobarbituric acid (Clin. Chim. Acta, 112, 197-204 (1981)). An enzymatic method is currently proposed as a method for assaying a glyated protein in which the procedure is carried out in a simpler and more cost-effective manner within a shorter period of time with higher accuracy compared to the above-described methods (Japanese Patent Publication (kokoku) No. 33997/1993 (Hei5-33997), Japanese Patent Laid-Open No. 127895/1999 (Hei11-127895), and WO 97/13872).

[0005] In these enzymatic methods, a glyated protein is decomposed with protease and a fructosyl amino acid oxidase is allowed to act on a liberated glyated amino acid to assay the produced hydrogen peroxide. Examples of oxidases disclosed as usable in the enzymatic assay methods include an oxidase produced from a bacteria belonging to the genus *Corynebacterium* (Japanese Patent Publication (kokoku) Nos. 33997/1993 (Hei5-33997) and 65300/1994 (Hei6-65300)), an oxidase produced from a fungus belonging to the genus *Aspergillus* (Japanese Patent Laid-Open No. 155780/1991 (Hei3-155780)), an oxidase produced from a fungus belonging to the genus *Gibberella* (Japanese Patent Laid-Open No. 289253/1995 (Hei7-289253)), an oxidase produced from a fungus belonging to the genus *Fusarium* (Japanese Patent Laid-Open Nos. 289253/1995 (Hei7-289253) and 154672/1996 (Hei8-154672)), an oxidase produced from a fungus belonging to the genus *Penicillium* (Japanese Patent Laid-Open No. 336386/1996 (Hei8-336386)), and a ketoamine oxidase (Japanese Patent Laid-Open No. 192193/1993 (Hei5-192193)). These enzymes effectively react with a glyated amino acid. However, the above enzymes do not react with a glyated peptide in which an amino group of a peptide has been glyated.

[0006] At present, various glyated proteins are used as an index for diagnosing diabetes. Examples of such glyated proteins include those in which an  $\epsilon$ -amino group of an internal lysine residue in a protein is glyated (for example, glyated albumin) and those in which an  $\alpha$ -amino group of an amino acid at the amino terminus of the protein is glyated (for example, glyated hemoglobin (HbA1c)). Currently, however, some glyated proteins cannot be decomposed to quantitatively liberate a glyated amino acid even with the use of conventional protease. In addition, the above-described fructosyl amino acid oxidase, which is currently employed, has a high reactivity to a liberated glyated amino acid although it does not substantially react with a glyated peptide. Thus, the above enzymatic method is not always accurate.

[0007] For example, glyated hemoglobin (HbA1c) is produced by glyating an  $\alpha$ -amino group of an amino acid at the amino terminus of hemoglobin  $\beta$ -subunit. However, even though various proteases react with the glyated protein, the  $\alpha$ -glyated amino acid (an  $\alpha$ -amino group of the amino acid is glyated) cannot be liberated. Thus, even with the use of the above-described fructosyl amino acid oxidase, glyated hemoglobin (HbA1c) cannot be assayed.

[0008] At present, methods proposed for assaying glyated hemoglobin (HbA1c) include a method in which glyated hemoglobin is directly assayed in that state by electrospray-ionization mass spectrometry (Clinical Test, 42, 340-343 (1997)), a method in which endoproteinase Glu-C is allowed to act on glyated hemoglobin, a liberated  $\alpha$ -glyated hexapeptide derived from  $\beta$ -subunit ( $\alpha$ -amino group of an amino acid at the amino terminus of hexapeptide is glyated)

is fractionated by reversed phase high performance liquid chromatography, and the content thereof is determined through mass spectrometry analysis to assay (Clin. Chem., 43, 1994-1951 (1997)), and the like. However, a highly sensitive and expensive assay device is necessary for these methods, and the operation of the assay is complicated, costly, and time-consuming.

[0009] The object of the present invention is to overcome the drawbacks of the conventional methods for assaying a glycosylated protein and to provide a novel method for assaying a glycosylated protein by a simple procedure based on a principle different from that of the conventional enzymatic methods in a cost-effective manner within a short period of time with high accuracy.

## DISCLOSURE OF THE INVENTION

[0010] We have conducted concentrated studies to attain the above object and as a result have found that, by a certain type of protease treatment, an  $\alpha$ -glycosylated peptide having a specific number of amino acid residues (a glycosylated peptide in which an  $\alpha$ -amino group of an amino acid at the amino terminus of the peptide has been glycosylated), particularly an  $\alpha$ -glycosylated dipeptide (a glycosylated dipeptide in which an  $\alpha$ -amino group of an amino acid at the amino terminus of the dipeptide has been glycosylated), is efficiently liberated from a glycosylated protein and that an enzyme in which a fructosyl amino acid oxidase produced from microorganisms is modified acts on the liberated  $\alpha$ -glycosylated peptide, especially an  $\alpha$ -glycosylated dipeptide in a specific manner, to produce hydrogen peroxide. Further, an  $\alpha$ -glycosylated peptide liberated from the glycosylated protein can be assayed by HPLC or using the above-described oxidase, and as a result, a glycosylated protein can be assayed by a simple procedure, within a short period of time, and with high accuracy. This has led to the completion of the present invention.

[0011] The present invention provides a method for assaying the presence and/or amount of a glycosylated protein in a sample, and a reagent kit for assaying, which is used in the above method. In the assay method, a sample containing or capable of containing a glycosylated protein is treated with protease, and when a glycosylated protein is present, a glycosylated peptide, preferably an  $\alpha$ -glycosylated peptide, particularly preferably an  $\alpha$ -glycosylated dipeptide, is liberated therefrom, and an oxidase having an activity to produce hydrogen peroxide upon reacting with the glycosylated peptide is allowed to act on those liberated glycosylated peptides, thereby assaying the resultant hydrogen peroxide and the like, or alternatively, the liberated glycosylated peptide is assayed by HPLC.

[0012] Another aspect of the present invention provides a method for assaying the presence and/or amount of a glycosylated peptide in a sample by allowing an oxidase having an activity to produce hydrogen peroxide upon reacting with a glycosylated peptide to react with a sample containing or capable of containing the glycosylated peptide to assay the resulting hydrogen peroxide and the like.

[0013] More specifically, the present invention provides the following (1) to (12).

(1) A method for assaying the presence and/or amount of a glycosylated protein in a sample, wherein the sample is treated with protease, followed by treatment with an oxidase having an activity to produce hydrogen peroxide upon reacting with a glycosylated peptide to assay the presence and/or amount of a generated product or consumed substance by the reaction.

(2) The method for assaying the presence and/or amount of a glycosylated protein according to (1) above, wherein the protease is at least one protease selected from proteases produced by microorganisms belonging to the genus *Aspergillus*, *Saccharomyces*, or *Bacillus*.

(3) The method for assaying the presence and/or amount of a glycosylated protein according to (1) above, wherein the glycosylated peptide is an  $\alpha$ -glycosylated peptide.

(4) The method for assaying the presence and/or amount of a glycosylated protein according to (3) above, wherein a peptide portion of the  $\alpha$ -glycosylated peptide is a short chain peptide having 2 to 6 amino acids.

(5) The method for assaying the presence and/or amount of a glycosylated protein according to (3) above, wherein the  $\alpha$ -glycosylated peptide is fructosyl valyl histidine.

(6) The method for assaying the presence and/or amount of a glycosylated protein according to (1) above, wherein the product to be assayed is hydrogen peroxide.

(7) A method for assaying the presence and/or amount of a glycosylated protein in a sample, wherein the sample is treated with protease, and the presence or absence, and/or amount of liberation of fructosyl valyl histidine is then assayed by HPLC.



(8) A method for assaying the presence and/or amount of a glycosylated peptide in a sample, wherein the sample is treated with an oxidase having an activity to produce hydrogen peroxide upon reacting with the glycosylated peptide to assay the presence and/or amount of a generated product or consumed substance produced by the reaction.

(9) A reagent kit for assaying a glycosylated protein in a sample, comprising the following components:

- (i) protease;
- (ii) an oxidase having an activity to produce hydrogen peroxide by reacting with a glycosylated peptide; and
- (iii) a reagent for assaying hydrogen peroxide.

(10) The reagent kit for assaying a glycosylated protein in a sample according to (9) above, wherein the glycosylated peptide is an  $\alpha$ -glycosylated peptide.

(11) The reagent kit for assaying a glycosylated protein in a sample according to (10) above, wherein a peptide portion of the  $\alpha$ -glycosylated peptide is a short chain peptide having 2 to 6 amino acids.

(12) The reagent kit for assaying a glycosylated protein in a sample according to (10) above, wherein the  $\alpha$ -glycosylated peptide is fructosyl valyl histidine.

[0014] This specification includes part or all of the contents disclosed in the specification and/or drawings of Japanese Patent Application No. 280,941/1999 (Hei11-280941), which is a priority document of the present application.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[0015]

Fig. 1 shows a result of assay of an  $\alpha$ -glycosylated dipeptide (fructosyl valyl histidine) in a sample using a method of the present invention.

Fig. 2 shows a result of assay of glycosylated hemoglobin (HbA1c) in a sample using the method of the present invention.

#### BEST MODE FOR CARRYING OUT THE INVENTION

[0016] The present invention will now be described in detail. As described above, the glycosylated protein according to the present invention may be of any type so long as it is produced through nonenzymatic binding between a protein and aldose such as glucose. For example, organism-derived glycosylated protein includes glycosylated albumin and glycosylated hemoglobin (HbA1c) and the present invention is preferably used in the assay of, for example, glycosylated hemoglobin (HbA1c). Further, glycosylated protein is included in foods at large, for example, juice, candies, seasonings, and powdery foods. Any sample can be utilized as a sample containing the glycosylated protein of the present invention so long as the glycosylated protein described above is contained. Examples include hair and body fluids such as blood and saliva in an organism as well as the above-described foods. These samples may be subjected to assay as they are or after filtration, dialysis or the like. Further, for example, the glycosylated protein to be assayed may be adequately concentrated, extracted, or diluted with water, a buffer or the like.

[0017] In the present invention, protease is first utilized to liberate a glycosylated peptide from a glycosylated protein. "Protease" as used herein refers to an enzyme having a general protease activity and/or a peptidase activity. Any enzyme may be used as protease so long as the enzyme can react with the glycosylated protein to liberate the glycosylated peptide, and suitable enzymes can be appropriately selected depending on the type of the subject glycosylated protein. Suitable enzymes include, for example, protease and peptidase such as proteinase K, pronase E, thermolysin, subtilisin, carboxypeptidase B, cathepsin, carboxypeptidase, endoproteinase Glu-C, papain, and aminopeptidase. In the present invention, protease capable of efficiently liberating a glycosylated peptide on which the oxidase used in the present invention, described below, can easily act is desirable. Particularly preferably, protease for efficiently liberating an  $\alpha$ -glycosylated dipeptide includes protease derived from a fungus belonging to the genus *Aspergillus*, for example, "Molsin", "AO Protease", or "Peptidase" (commercially available from Seishin Corporation), carboxypeptidase Y derived from a yeast belonging to the genus *Saccharomyces*, and protease derived from a bacteria belonging to the genus *Bacillus*, such as Protin-P (commercially available from Daiwa Kasei). The above proteases can be used alone or in a combination of two or more.

[0018] Any conditions may be employed for treating a sample so long as the protease acts on the glycosylated protein to be assayed to efficiently liberate the glycosylated peptide within a short period of time. A amount of protease to be used

is appropriately determined depending on the content, treatment conditions and the like of a glycosylated protein contained in the sample. As one example, 0.5 to 50 mg/mL, preferably 1 to 20 mg/mL, of protease derived from a fungus belonging to the genus *Aspergillus* (for example, Molsin, commercially available from Seishin Corporation) is added. Other protease may be appropriately added as required. When treating with protease, pH may be left unadjusted; however, in order to render the pH value to be suitable for the activity of the protease to be used, pH may be adjusted to 2 to 9, preferably 3 to 8, with the aid of an adequate pH adjuster, for example, hydrochloric acid, acetic acid, sulfuric acid, sodium hydroxide, or potassium hydroxide. A treatment temperature may be, for example, 20 to 50°C, and it may be in a higher temperature range of 45 to 70°C depending on the type of enzyme to be used. A treatment time may be of any duration so long as it is long enough to decompose a glycosylated protein, and may be 1 to 180 minutes, preferably 2 to 60 minutes. The resulting reaction mixture may be used as is or appropriately heated, centrifuged, concentrated, diluted or the like, if necessary.

[0019] The liberated glycosylated peptide of the present invention, which is prepared through protease treatment of a sample, includes any type of glycosylated peptide so long as it is a glycosylated peptide that can be obtained by treating a glycosylated protein with the protease and that the oxidase used in the present invention, described below, can easily act thereon, with an  $\alpha$ -glycosylated peptide being preferred, for example, a short chain  $\alpha$ -glycosylated peptide having 2 to 6 amino acids of the peptide or the like. An  $\alpha$ -glycosylated dipeptide, for example, fructosyl valyl histidine (hereinafter abbreviated to "fructosyl Val-His" or "Fru-Val-His") or the like is particularly preferred. Organisms or foods include, for example, a liberated glycosylated peptide prepared through decomposition of a glycosylated protein in an organism or in the production process of foods, respectively, and a glycosylated peptide prepared by binding between a liberated peptide through decomposition of a protein and a saccharide and the like. These are also included in the liberated glycosylated peptide of the present invention.

[0020] Subsequently, the glycosylated peptide is assayed. Any method can be employed in the present invention as long as the glycosylated peptide can be assayed. Preferred methods for assaying a glycosylated peptide by a simple procedure, in a cost-effective manner, within a short period of time, and with high accuracy include, for example, a method in which an oxidase is allowed to act and a method in which HPLC is utilized.

[0021] A method for causing the oxidase to act of the present invention will now be described. The oxidase is allowed to act on the glycosylated peptide, and by assaying a generated product or consumed substance by the reaction, the glycosylated peptide is assayed in an enzymatic manner. Any enzyme can be employed as the oxidase used in the present invention so long as the enzyme (hereinafter referred to as "the oxidase of the present invention") can act in a specific manner on a glycosylated peptide, preferably an  $\alpha$ -glycosylated peptide, particularly preferably a short chain  $\alpha$ -glycosylated peptide such as an  $\alpha$ -glycosylated dipeptide, to catalyze a reaction for generating hydrogen peroxide. For example, a novel oxidase such as a glycosylated peptide oxidase can be used.

[0022] In general, a microorganism for producing the oxidase of the present invention can be obtained by screening the natural world or the enzyme of the present invention can be obtained by searching from animals or plants. In addition, those prepared through gene recombination of enzymes obtained by searching can be suitably used. Meanwhile, a conventional fructosyl amino acid oxidase may be modified to obtain the oxidase of the present invention. Examples of the conventional fructosyl amino acid oxidase include the oxidase produced from a bacteria belonging to the genus *Corynebacterium* (Japanese Patent Publication (kokoku) Nos. 33997/1993 (Hei5-33997) and 65300/1994 (Hei6-65300)), the oxidase produced from a fungus belonging to the genus *Aspergillus* (Japanese Patent Laid-Open No. 155780/1991 (Hei3-155780)), the oxidase produced from a fungus belonging to the genus *Gibberella* (Japanese Patent Laid-Open No. 289253/1995 (Hei7-289253)), the oxidase produced from a fungus belonging to the genus *Fusarium* (Japanese Patent Laid Open Nos. 289253/1995 (Hei7-289253) and 154672/1996 (Hei8-154672)), the oxidase produced from a fungus belonging to the genus *Penicillium* (Japanese Patent Laid-Open No. 336386/1996 (Hei8-336386)), and the ketoamine oxidase (Japanese Patent Laid-Open No. 192193/1993 (Hei5-192193)).

[0023] In order to obtain the oxidase of the present invention through modification of the conventional fructosyl amino acid oxidase and the like, a microorganism capable of producing the conventional fructosyl amino acid oxidase and the like is irradiated with ultraviolet ray, X-ray, radiation or the like or is contacted with a mutagen such as ethyl methane sulfonate, N-methyl-N'-nitro-N-nitrosoguanidine, or nitrous acid to perform mutation. The microorganism for producing the oxidase of the present invention is selected from the resulting mutated microorganisms.

[0024] In general, however, the oxidase of the present invention can be prepared through introduction of mutation into genes of the conventional fructosyl amino acid oxidase or the like (hereinafter referred to as wild-type genes). Any wild-type genes may be used for introduction of mutation so long as the gene is a wild-type gene of the fructosyl amino acid oxidase or an analogous oxidase and can produce the oxidase of the present invention through introduction of mutation.

[0025] The above wild-type gene can be obtained by cloning naturally-occurring genes derived from organisms capable of producing a fructosyl amino acid oxidase, an analogous oxidase or the like, preferably naturally-occurring genes derived from microorganisms. Cloning is carried out by first extracting chromosome DNA or mRNA from organisms producing the oxidase in accordance with a conventional method, for example, a method described in Current

Protocols in Molecular Biology (WILEY Interscience, 1989). Further, mRNA can be used as a template to synthesize cDNA. A library of the thus obtained chromosome DNA or cDNA is produced. Subsequently, suitable probe DNA is synthesized based on the amino acid sequence of the oxidase and the like to screen the library of DNA or cDNA using the probe DNA. Alternatively, suitable primer DNAs are prepared based on the amino acid sequence of the peptide, DNA containing the subject gene fragment is amplified by a suitable polymerase chain reaction (PCR) such as a 5'RACE or 3'RACE method, and the amplified DNAs are linked to each other to obtain a DNA containing a full length of wild-type gene. As a further example, a method exists in which isolation is carried out in accordance with a conventional method from *Escherichia coli* DH5 $\alpha$  (pFA5) (FERM BP-6182) that maintains plasmid DNA coding for a wild-type gene of the present invention derived from a bacteria belonging to the genus *Corynebacterium*, as a generally available source for genes.

[0026] Methods for introducing mutation into the wild-type genes include a method in which a wild-type gene is contacted with a mutating agent, for example, an alkylating agent such as nitrosoguanidine, an acridine dye, hydroxylamine, nitrous acid, sulfurous acid, 5-bromouracil, or benzopyrene. In addition, a method for introducing mutation utilizing, for example, ultraviolet radiation, transposon, a cassette-type mutation, chimera gene preparation, PCR, or DNA shuffling can be extensively employed. The wild-type genes for introducing mutation may be genes that were inserted into suitable vector DNA, i.e., recombinant DNA. In this case, recombinant DNA after mutation is purified by ethanol precipitation and the like. The resulting mutated genes can be expressed by genetic transformation or genetic transduction of a host cell utilizing the recombinant DNA. The strain for producing the oxidase of the present invention is selected from a large number of strains maintaining mutated genes.

[0027] A method for selecting the subject microorganisms or strains include a method utilizing, as a substrate, an  $\alpha$ -glycated peptide, preferably a short chain  $\alpha$ -peptide such as an  $\alpha$ -glycated dipeptide, an  $\alpha$ -glycated tripeptide, or an  $\alpha$ -glycated tetrapeptide. An example thereof includes a method utilizing fructosyl Val-His or the like as an  $\alpha$ -glycated dipeptide. To the reaction solution containing this substrate is added an enzyme extract prepared by fragmentation or bacteriolysis of bacterial cells of microorganisms or strains to be assayed, or an enzyme extract prepared from the supernatant thereof separated by centrifugation to conduct reaction. The resulting hydrogen peroxide is colored with a commonly employed coloring substrate for hydrogen peroxide described below, thereby selecting the microorganisms or strains for producing the oxidase of the present invention. The enzyme extract may be used in that state, or may be optionally concentrated or diluted. The amount of oxygen decrease due to the enzyme reaction can be measured with an oxygen electrode. In selection, an enzyme reaction may be carried out in a test tube. Examples which may be appropriately adopted include a method in which the reaction is performed in a 96-well microplate, a method in which the reaction is performed by coating or permeating a reaction reagent on a membrane onto which an enzyme extract has been adsorbed, and a method in which the reaction is performed by superimposing a membrane having a reaction reagent coated thereon on a membrane having an enzyme extract adsorbed thereon. Alternatively, selection can be carried out in an efficient simple manner by mixing a plurality of strains to perform several steps of selection.

[0028] As described above, *Escherichia coli* (*E. coli*) DH5 $\alpha$ (pFP1) can be specifically exemplified as a strain for producing the oxidase of the present invention, which was prepared by modifying a conventional fructosyl amino acid oxidase produced from a bacteria belonging to the genus *Corynebacterium* (Japanese Patent Publication (kokoku) Nos. 33997/1993 (Hei5-33997) and 65300/1994 (Hei6-65300)). *Escherichia coli* (*E. coli*) DH5 $\alpha$ (pFP1) was deposited at the RESEARCH INSTITUTE OF BIOSCIENCE AND HUMAN-TECHNOLOGY (1-1-3, Higashi, Tsukuba, Ibaraki, Japan) as of September 22, 1999 under the accession number FERM BP-7297.

[0029] The oxidase of the present invention is obtained from tissues of animals, plants and the like containing the enzyme or the microorganisms producing the enzyme by, for example, a conventional extraction method. For example, the oxidase of the present invention is produced using microorganisms producing the oxidase of the present invention in the manner described below. The microorganisms may be cultured in accordance with a conventional solid culturing technique. Preferably, however, they are cultured in accordance with a liquid culturing technique as much as practically possible. Any medium can be used in culturing as long as a carbon source, a nitrogen source, an inorganic substance, and other nutritive sources are adequately contained therein. The medium may be either synthetic or naturally-occurring, and as long as the medium can efficiently produce the subject enzyme, any medium may be used. An assimilable carbon compound suffices for the carbon source and examples thereof include glucose, starch hydrolysate, glycerin, fructose, and molasses. Any usable nitrogen compound suffices for a nitrogen source and examples thereof include a yeast extract, a peptone, a meat extract, corn steep liquor, soybean flour, a Malt extract, an amino acid, ammonium sulfate, and ammonium nitrate. Inorganic substances include, for example, various salts such as a common salt, potassium chloride, magnesium sulfate, manganese chloride, iron (I) sulfate, potassium (I) phosphate, potassium (II) phosphate, sodium carbonate, and calcium chloride. In addition, vitamins, antifoaming agents and the like may be optionally added. A substrate on which the oxidase of the present invention acts or an analogous material thereof, for example, glycated peptides, fructosyl amino acid, partial decomposition product of a glycated protein, glycated hemoglobin, glycated albumin, glycated peptide that was artificially glycated through coheating with saccharide, or a glycated protein may be added to increase the amount of the subject enzyme produced. These nutritive sources and substances

to be added may be used alone or in combination. Culture conditions vary depending on microorganisms to be cultured. For example, culturing is carried out with the initial pH of the medium being adjusted to 5 to 10, at the culturing temperature of 20 to 40°C, for the culturing time of 10 to 50 hours, preferably 15 to 25 hours, by means of submerged culturing with aerating stirring, shake culturing, stationary culturing or the like.

[0030] After the completion of culturing, a conventional means for collecting an enzyme can be employed in order to collect the oxidase of the present invention from the culture product. When the enzyme is present in the strain, preferably, a strain is separated from the cultured product through an operation such as filtration and centrifugation to collect the enzyme from the strain. For example, a method for cleaving strains using a conventional cleavage means such as an ultrasonic crusher, a French press, or DYNO-Mill, a method for dissolving a cell wall of a strain using a cell wall lytic enzyme such as lysozyme, or a method for extracting an enzyme from the strain using a surfactant such as Triton X-100 may be adopted by itself or in combination. Subsequently, an insoluble substance is removed by filtration or centrifugation to obtain an enzyme extract. In order to optionally isolate and purify the oxidase of the present invention from the resultant extract, a nucleic acid is removed therefrom with the aid of streptomycin sulfate, protamine sulfate, manganese sulfate or the like, and ammonium sulfate, alcohol, acetone or the like is then added thereto. The mixture is fractionated and a precipitate is collected, thereby obtaining a crude enzyme. In order to obtain a more purified enzyme sample, for example, gel filtration utilizing Sephadex, ultragel, or biogel, an adsorption-elution method utilizing an ion exchanger, hydroxyapatite or the like, affinity chromatography, or fractionation utilizing a molecular sieve membrane, hollow fiber membrane or the like are appropriately selected or performed in combination to obtain an enzyme sample having a desired level of purity. When the enzyme is present outside the strain, in accordance with a conventional method, a culture solution is collected and concentrated after the separating operation of the strain to subject the culture solution to the above various purifying methods.

[0031] A titer of the oxidase of the present invention can be measured by, for example, the following method. It can also be measured by other methods and the measuring method is not limited to that described below.

#### (1) Preparation of reagents

[0032] Reagent 1 (R1): 1.0 kU of peroxidase (TYPE III, Toyobo Co.) and 100 mg of 4-aminoantipyrine (TOKYO KASEI KOGYO CO., LTD.) are dissolved in 0.1 M potassium phosphate buffer (pH 8.0) to fix the solution at a constant volume of 1 L.

[0033] Reagent 2 (R2): 500 mg of TOOS (N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine, DOJINDO LABORATORIES) is dissolved in ion-exchange water to fix the solution at a constant volume of 100 mL.

[0034] Reagent 3 (R3): 1.25 g of fructosyl Val-His (MW 416, a production process thereof is described below) is dissolved in ion-exchange water to fix the solution at a constant volume of 10 mL.

#### (2) Assay

[0035] 100  $\mu$ L of R2 is added to 2.7 mL of R1, and 100  $\mu$ L of an enzyme solution containing the oxidase of the present invention is added thereto, followed by mixing. The mixture is preheated at 37°C for 5 minutes. 100  $\mu$ L of R3 is then added and the mixture is thoroughly mixed. Thereafter, a spectrophotometer (U-2000A, Hitachi) is used to measure changes in absorbance at 555 nm during the reaction at 37°C for 5 minutes. A control liquid is prepared according to the same procedure as above except for the addition of 100  $\mu$ L of ion-exchange water instead of 100  $\mu$ L of R3. A previously prepared standard solution of hydrogen peroxide is used to provide a graph showing a correlation between the amount of hydrogen peroxide and the amount of a resulting dye (absorbance), from which the amount of hydrogen peroxide corresponding to the change in absorbance is determined. From the obtained value, an activity unit in the enzyme solution is determined. The amount of enzyme that produces 1  $\mu$ mol of hydrogen peroxide per one minute is determined as 1 U.

[0036] The thus-obtained oxidase of the present invention acts on a glycosylated peptide in a specific manner and has a property that produces hydrogen peroxide. Thus, not only can the oxidase of the present invention enzymatically assay the glycosylated peptide contained in an organism, food or the like, but it can also enzymatically assay the liberated glycosylated peptide prepared by treating glycosylated protein in an organism with protease. Therefore, it is suitable for use as a reagent for assaying the glycosylated protein of the present invention.

[0037] The glycosylated peptide liberated through protease treatment is acted on by the oxidase of the present invention. The oxidase of the present invention used may be added, amount for example, to bring the final concentration of the reaction mixture to 0.1 to 50 U/mL, preferably 1 to 10 U/mL, although it depends on the amount of the glycosylated peptide in the mixture. When reacting, the range of pH value is, for example, preferably 3 to 11, particularly preferably 5 to 9. It is preferable to adjust the pH value with the aid of a buffer to be suitable for the assay of the present invention considering the optimal pH value of the oxidase of the present invention, although the pH value is not limited to the optimal value so long as it is a pH that is capable of reacting. The methods for adjusting pH are not particularly limited,

and examples of buffers include N-[tris(hydroxymethyl)methyl]glycine, phosphate, acetate, carbonate, tris(hydroxymethyl)-aminomethane, borate, citrate, dimethylglutamate, Tricine, and HEPES. If necessary, the pH value of the reaction mixture after protease treatment is appropriately adjusted to the above pH using a buffer. The reaction time is, for example, 1 to 120 minutes, preferably 1 to 30 minutes, although it depends on the amount of the glydated peptide used as a substrate, and the reaction time may be of any duration so long as it is long enough for the oxidase of the present invention to react with the peptides. The reaction temperature is, for example, 20 to 45°C, and the temperature to be employed in a conventional enzyme reaction can be appropriately selected.

[0038] According to the present invention, the oxidase of the present invention acts on the liberated glydated peptide, and by assaying the generated product or consumed substance by the reaction, the glydated peptide is assayed. Substances (products) produced from the glydated peptide by the reaction of the oxidase of the present invention include, for example, peptides, hydrogen peroxide, and glucosone. On the other hand, substances to be consumed (consumed substances) include oxygen molecules. These products and consumed substances can be assayed using respective measuring methods. For example, methods for assaying oxygen molecules include an electrical method using an oxygen electrode and methods for assaying peptides include the assay by separation utilizing a reversed-phase HPLC. Preferably, examples of methods that enable assaying within a short period of time by a simple procedure include a method for assaying hydrogen peroxide.

[0039] Hydrogen peroxide produced by the reaction of the oxidase of the present invention may be assayed by any method and examples thereof include an electrical method using an oxygen electrode, and preferably an enzymatic method using peroxidase and an adequate coloring substrate. For example, in the present invention, the assay is preferably carried out in an enzymatic manner within a short period of time by a simple procedure. Examples of reagents for assaying hydrogen peroxide by the enzymatic method of the present invention include a reagent comprising 5 to 500 mM, preferably 50 to 100 mM of buffer (pH of 4 to 10 being preferred), 0.01 to 50 mM, preferably 0.1 to 20 mM of 4-aminoantipyrine as a coloring substrate, and 0.1 to 50 U/mL, preferably 1 to 20 U/mL of peroxidase. Buffers used in the present invention include, for example, N-[tris(hydroxymethyl)methyl]glycine, phosphate, acetate, carbonate, tris(hydroxymethyl)-aminomethane, borate, citrate, dimethylglutamate, Tricine, and HEPES. Coloring substrates include, in addition to 4-aminoantipyrine, for example, ADOS (N-ethyl-N-(2-hydroxy-3-sulfoethyl)-m-anisidine), ALOS (N-ethyl-N-(2-hydroxy-3-sulfoethyl)aniline),

10-(carboxymethylaminocarbonyl)-3,7-bis(dimethylamino)-phenothiazine (DA-67), and N-(carboxymethylaminocarbonyl)-4,4'-bis(dimethylamino)-diphenylamine (DA-64). If necessary, various additives, for example, surfactants (e.g., Triton X-100, Bridge 35, Tween 80, cholate), reducing agents (e.g., dithiothreitol, mercaptoethanol, and L-cysteine), bovine serum albumin, and saccharides (e.g., glycerin, lactose, and sucrose), may be appropriately added as solubilizing agents, stabilizers or the like within a range which does not impair the object of the present invention.

[0040] In general, the step of producing hydrogen peroxide by the reaction of the oxidase is preferably carried out concurrently with the assay of hydrogen peroxide. In the present invention, therefore, the oxidase of the present invention is preferably added to the reagents for assaying the hydrogen peroxide in an amount of, for example, 0.1 to 50 U/mL, preferably 1 to 10 U/mL. These reagents for assaying may be used in a dried or dissolved state. Alternatively, the reagents may be used after being impregnated into a carrier in a thin film form, such as an impregnable paper sheet. The enzymes used in the assay reagent of the present invention can be repetitiously used by immobilization in accordance with a conventional method. The assay temperature is, for example, 20 to 45°C, and a temperature employed in a conventional enzyme reaction can be appropriately selected. The time required for the assay can be appropriately selected depending on various assay conditions and, for example, is preferably 0.1 to 60 minutes, particularly preferably 1 to 10 minutes, in the present invention. The level of color development of the assay reagent (change in absorbance) is measured with a spectrophotometer, and the measured value is compared with a standard absorbance, thereby assaying a glydated peptide or glydated protein contained in a sample. A conventional autoanalyzer can be used in the assay.

[0041] An assay reagent kit for assaying a glydated protein in a sample according to the present invention comprises protease used for liberating a glydated peptide from the glydated protein, the oxidase of the present invention, a reagent for assaying hydrogen peroxide, and the like. Specific formulations of the respective components can be as described above. The above components may be separately stored and used or the oxidase of the present invention and the reagent for assaying hydrogen peroxide may be stored and used in combination. In the present invention, when a glydated protein is assayed using the reagent kit, for example, a step of liberating a glydated peptide and a step of assaying the liberated glydated peptide may be separately performed in two steps, or alternatively, those components may be combined to perform the above steps consecutively in one step.

[0042] Next, a method for assaying a liberated glydated peptide by HPLC will be described. A protease treatment mixture liquid containing a liberated glydated peptide is used in that state for the assay by HPLC. Alternatively, the mixture is optionally filtrated by centrifugation or through membrane and the filtrate is appropriately concentrated or diluted for the assay by HPLC. Any HPLC may be used for the HPLC in the present invention so long as the HPLC can assay the glydated peptide. For example, columns for reversed phase HPLC usable herein include CAPCEL-PAK

C-18 (Shiseido Co., Ltd.), TSKgel ODS80Ts (Tosoh Corp.), and Shodex RSPak RP18-415 (Showa Denko K.K.) and columns for ion-exchange HPLC include TSKgel SP-2SW and TSKgel CM-2SW (Tosoh Corp.). After protease treatment, reaction mixture is adsorbed on these columns, and an eluent is used to elute a subject glycosylated peptide. Any eluent may be used so long as it is suitable for the assay in the present invention, and examples thereof include, in the case of reversed phase column, a mixed solution of acetonitrile and water containing trifluoroacetic acid, a mixed solution of a phosphate buffer and acetonitrile, and a mixed solution of an aqueous solution of ammonia and acetonitrile, and in the case of ion-exchange columns, a mixed solution of a phosphate buffer and a NaCl solution and a mixed solution of an acetate buffer and acetonitrile. Stepwise elution or gradient elution may be carried out using these eluents. Preferable eluents include, for example, a gradient eluent of 0.1% TFA (trifluoroacetic acid)/water-0.1% TFA/30% acetonitrile. Preferably, in the present invention, a column, an eluent, the elution conditions (e.g., elution method, flow rate of an eluent, and temperature) and the like are appropriately combined and the conditions are set up so that the elution peak of the subject  $\alpha$ -glycosylated peptide is separated satisfactorily from that of other components at an optimal level.

[0043] As a method for detecting a glycosylated peptide eluted by an eluent, any method can be utilized as long as the glycosylated peptide can be detected therewith. For example, usable methods include a method for detecting absorbance at a wavelength of 210 nm, 215 nm, or the like, a method for determining the peak of the subject molecular weight by fractionating each detected peak, followed by mass spectrometry, a method utilizing thin-layer chromatography, and a method in which an elution fraction that was fractionated over time is subjected to colorimetric measurement with ninhydrin or saccharide coloring. As one example, when a method for detecting absorbance is employed, the area of the elution peak of the glycosylated peptide detected by a monitor is calculated and the calculated value is compared with the area of the elution peak of a standard substance. Thus, the amount of the glycosylated peptide and the glycosylated protein can be assayed.

[0044] The present invention will be described in more detail with reference to a reference example and examples, although the technical scope of the present invention is not limited to these examples only.

#### Reference Example (production of glycosylated dipeptide)

[0045] The  $\alpha$ -glycosylated dipeptide used in the present invention was produced in accordance with the following method. 7.0 g (27.6 mmol) of commercially available dipeptide (valyl histidine (Val-His), BACHEM, Switzerland) was dissolved in 14 mL of water, 5.8 mL of acetic acid was added and dissolved at about 50°C, and the mixture was clarified. Subsequently, 120 mL of ethanol was added thereto and mixed, 14 g (77.8 mmol) of glucose was then added, and the mixture was thoroughly mixed. Thereafter, the mixture was heated in a hermetically sealed container at 80°C for 6 hours while sporadically stirring. The reaction solution was browned over time. An aliquot was taken from the reaction solution over time, adequately diluted, and subjected to analysis by reversed phase high performance liquid chromatography, thin-layer chromatography, or mass spectrometry to assay the production of the subject glycosylated dipeptide. In general, a glycosylated dipeptide can be obtained in a high yield by heating for 6 to 10 hours. Next, the reaction solution was collected and concentrated to fifteen-fold to thirty-fold using a rotary evaporator. The concentrate was adsorbed on a silica gel-column (bed volume: 2,000 mL) that had been equilibrated with 99.5% ethanol and washed with 2 bed volumes of 99.5% ethanol, and impurities such as unreacted glucose were removed. Subsequently, elution was carried out using 3 bed volumes of 95% ethanol, 3 bed volumes of 90% ethanol, 3 bed volumes of 85% ethanol, and 3 bed volumes of 80% ethanol, in that order. Each elution fraction was analyzed by thin-layer chromatography, reverse phase high performance liquid chromatography and the like to collect a 95 to 90% ethanol elution fractions containing a subject fructosyl Val-His. The collected fractions were concentrated to dryness using a rotary evaporator to obtain about 3 g of partially purified product. As a result of mass spectrometry analysis, the molecular weight of the purified product was 416, which was congruous with the molecular weight of fructosyl Val-His, and the structure thereof was confirmed using nuclear magnetic resonance spectrum analysis. In accordance with the conventional method, this partially purified product was subjected to adsorption and desorption using an ion-exchange resin, thereby enhancing the level of purity for use in subsequent experiments. Further, partially purified products of a glycosylated tripeptide, a glycosylated tetrapeptide, and a glycosylated hexapeptide were respectively obtained using a tripeptide, a tetrapeptide, and a hexapeptide, respectively, in the same manner as described above.

#### Example 1 (Liberating glycosylated dipeptide from glycosylated hexapeptide)

[0046] Treating glycosylated hemoglobin (HbA1c) with endoproteinase Glu-C results in liberation of an  $\alpha$ -glycosylated hexapeptide derived from the  $\beta$  subunit of glycosylated hemoglobin (HbA1c) (fructosyl Val-His-Leu-Thr-Pro-Glu) (Clin. Chem., 43, 1994-1951 (1997)). Therefore, the following experiment was carried out using fructosyl Val-His-Leu-Thr-Pro-Glu (PEPTIDE INSTITUTE, INC.), which is identical to the  $\alpha$ -glycosylated hexapeptide.

[0047] The  $\alpha$ -glycosylated hexapeptide (PEPTIDE INSTITUTE, INC.) was dissolved in water to prepare a 5 mM solution.

To 0.1 mL of this solution were added the following combinations of 0.01 mL of protease solution (20 mg/mL) and 0.09 mL of buffer (0.1 M) ((a) to (d)), followed by mixing to subject to protease treatment.

- (a) Carboxypeptidase Y (Oriental Yeast), a phosphate buffer (pH 6.5).
- (b) AO protease (commercially available from Seishin Corporation), a citric acid-disodium phosphate buffer (pH 6.0).
- (c) Peptidase (commercially available from Seishin Corporation), a citric acid-disodium phosphate buffer (pH 6.0).
- (d) Molsin (commercially available from Seishin Corporation), a citric acid-disodium phosphate buffer (pH 3.0).

**[0048]** The above mixture was subjected to reaction at 37°C for 60 minutes. Thereafter, the reaction mixtures were suitably concentrated or diluted respectively and assayed by HPLC. CAPCEL-PAK C-18 (Shiseido) was used for HPLC (reversed phase high performance liquid chromatography). Gradient elution was carried out using, as an eluent, 0.1% TFA (trifluoroacetic acid)/water-0.1 % TFA/30% acetonitrile. An  $\alpha$ -glycated dipeptide (fructosyl Val-His) was used as a standard substance. Further, the eluted glycated peptide was analyzed using thin-layer chromatography (a silica plate (Merck) was used, a developing solvent was n-butanol : acetic acid : water = 2 : 1 : 1, ninhydrin and ethanol-sulfuric acid were used for spot detection). As a result, in each combination of (a), (b), (c), and (d), the  $\alpha$ -glycated dipeptide (fructosyl Val-His) was liberated in the protease treatment reaction mixture. Further, each reaction mixture was subjected to amino acid analysis (amino acid analyzer L-8800, Hitachi) and mass spectrometry analysis (mass spectrometer Model M-80B, Hitachi). As a result of identification of the liberated amino acid residue and the measurement of the molecular weight thereof, it was found that, in each case, the  $\alpha$ -glycated hexapeptide (fructosyl Val-His-Leu-Thr-Pro-Glu) was cleaved in order from its carboxy terminus and/or internally cleaved and thereby decomposed into a shorter  $\alpha$ -glycated peptide. In the case of (a), while the liberation of Glu, Pro, Thr, and Leu residues from the carboxyl terminus was confirmed, the liberation of His residue was not confirmed. This indicates that it was shortened to fructosyl Val-His. Further, the analysis of the reaction mixture by mass spectrometry revealed that a major portion of the glycated peptide which was confirmed after treatment was fructosyl Val-His, and signals with molecular weights corresponding to fructosyl Val-His-Leu and fructosyl Val-His-Leu-Thr were found only at an insignificant level. In (b) and (c), a signal of fructosyl Val-His and a signal of a minor amount of fructosyl Val-His-Leu were found. In (d), only the signal of fructosyl Val-His was found.

#### Example 2 (Liberating glycated dipeptide from glycated protein)

**[0049]** Distilled water was added to a glycated hemoglobin (HbA1c) control (International Reagents) to prepare a solution of 8 g/dL (HbA1c content of about 10%). To 0.05 mL of this solution were added 0.01 mL of protease derived from the genus *Aspergillus* (Molsin, 20 mg/mL) and 0.04 mL of buffer (0.1 M, citric acid-disodium phosphate buffer, pH 3.0), followed by mixing. The mixed solution was treated with protease at 37°C for 180 minutes. Thereafter, the reaction mixture was subjected to centrifugal filtration using Microcon 3 (fraction molecular weight 3,000, Grace Japan K. K.) and the filtrate was diluted and then assayed by HPLC as described in Example 1. The liberation of fructosyl Val-His was confirmed and the glycated dipeptide was determined based on the area of the elution peak. The glycated protein was assayed using the measured value.

#### Example 3 (Obtaining the modified oxidase of the present invention)

##### (1) Preparation of template DNA

**[0050]** *Escherichia coli* DH5 $\alpha$ , which maintains a plasmid (pFA5) coding for a fructosyl amino acid oxidase genes derived from a bacteria belonging to the genus *Corynebacterium* (FERM BP-6182), was inoculated into 100 mL of LB-amp medium (1% bactotrypton, 0.5% bacto yeast extract, 0.5% sodium chloride, 50  $\mu$ g/mL ampicillin, pH 7.0), followed by shake culturing at 30°C for 24 hours to obtain a cultured product. 1.5 mg of pFA5 plasmid DNA was obtained from the cultured product in accordance with the method described in "Molecular Cloning (2nd. Edition, 1989)".

##### (2) Introduction of mutation

**[0051]** 30  $\mu$ g of pFA5 plasmid DNA was dissolved in 100  $\mu$ L of hydroxylamine solution (0.8 M hydroxylamine hydrochloride/0.1 M phosphate buffer, pH 6.0/1 mM EDTA). The solution was subjected to mutation treatment at 65°C for 2 hours and ethanol precipitation was performed by a conventional method to collect a precipitate. The precipitate was dissolved in a TE buffer (10 mM tris-hydrochloric acid buffer, pH 7.5/1 M EDTA), and *Escherichia coli* DH5 $\alpha$  strain was transformed in accordance with a method of D. M. Morrison (Method in Enzymology, 68, 326-331, 1979) and inoculated into a LB-amp agar medium (1% bactotrypton, 0.5% bacto yeast extract, 0.5% sodium chloride, 50  $\mu$ g/mL ampicillin,

1.5% (w/v) agarose, pH 7.0) to culture at 30°C for 24 hours.

### (3) Selection of producing microorganism

[0052] About 50,000 strains of colonies that were developed after 18 hours of culturing were transferred to Hybond-C which had been immersed in 30 mg/mL Lysozyme solution. On the then hand, Hybond-C that had been immersed in 50 mM fructosyl Val-His, 0.5 mg/mL peroxidase, 1.0 mg/mL 4-aminoantipyrine, 50 mg/mL TOOS, and 100 mM potassium phosphate buffer (pH 8.0) was prepared. The two Hybond-Cs were placed on each other in such a manner that the surfaces having bacterial cells thereon faced inward, and were then reacted at 37°C for about 30 minutes to 1 hour. Three strains with color development were selected and inoculated into 10 mL of LB-amp medium, and subjected to shake culturing at 30°C for 24 hours. Thereafter, the culture solution was fragmented by ultrasound treatment and centrifuged, and then the supernatant was assayed for its glycosylated peptide oxidase activity in the above-described manner. As a result, activity was detected in one strain. This strain was designated as *Escherichia coli* DH5 $\alpha$  (pFP1).

### (4) Enzyme production

[0053] The selected *Escherichia coli* DH5 $\alpha$  (pFP1) capable of producing the glycosylated peptide oxidase of the present invention was inoculated into 10 L of LB-amp medium and cultured while stirring with a jar fermenter at a amount of airflow of 1 L/min at a rate of stirring of 600 rpm at 30°C for 20 hours. The obtained 20 L of culture solution was concentrated to 5 L with the aid of an ultrafilter membrane with an MW of 50,000 (ASAHI KASEI CORP.) and 1 M potassium phosphate buffer (pH 8.0) was added thereto. Thereafter, bacterial cells were fragmented by DYNOMILL. The fragmented solution was centrifuged at 10,000 rpm for 15 minutes. The resultant supernatant was determined as a crude enzyme solution and subjected to purification in the manner described below.

[0054] Potassium chloride was added to the crude enzyme solution to bring the solution of 0.15 M and the solution was adsorbed on 2 L of DEAE-Sephacel column, which had been equilibrated by 50 mM potassium phosphate buffer (pH 8.0) containing 0.15 M potassium chloride. After washing with 2 L of the same buffer, elution was performed using a potassium phosphate buffer (a potassium chloride concentration: a linear gradient of 0.15 M to 0.50 M, pH 8.0). The activity of the obtained eluent was assayed based on the method for measuring a titer of the oxidase of the present invention, active fractions were then collected, the obtained enzyme solution was concentrated using an ultrafilter membrane with an MW of 6,000 (ASAHI KASEI CORP.), and dialyzed with 50 mM potassium phosphate buffer (pH 8.0) containing 16% ammonium sulfate. The product was then adsorbed onto a butyl TOYOPEARL column, which had been equilibrated by 50 mM potassium phosphate buffer (pH 8.0) containing 16% ammonium sulfate, washed with the same buffer, and eluted with 50 mM potassium phosphate buffer (an ammonium sulfate concentration: a linear gradient of 16% to 0%, pH 8.0) to collect active fractions. Subsequently, the enzyme solution was concentrated using an ultrafilter membrane with an MW of 6,000 (ASAHI KASEI CORP.) and dialyzed with 50 mM potassium phosphate buffer (pH 8.0). Thus, the subject enzyme solution was obtained.

#### Example 4 (Assay of $\alpha$ -glycosylated dipeptide using oxidase)

[0055] The following reagents were prepared for use in the assay of the glycosylated dipeptide.

Reagent A (coloring reagent)	
4-Aminoantipyrine (TOKYO KASEI KOGYO CO., LTD.)	0.2 mM
TOOS	0.2 mM
Peroxidase (Toyobo Co.)	14.3 U/mL
Potassium phosphate buffer (pH 8.0)	0.1 M

Reagent B (oxidase reagent)	
Oxidase obtained in Example 3	4 U/mL
Potassium phosphate buffer (pH 8.0)	0.02 M

[0056] The  $\alpha$ -glycosylated dipeptide, fructosyl Val-His obtained in accordance with the method described in Reference Example were used to prepare a 1.0 mmol/L solution. This solution was diluted and glycosylated dipeptide-containing samples with various concentrations (25, 50, 75, and 100  $\mu$ mol/L) were prepared. 2.1 mL of Reagent A was added to each of the glycosylated dipeptide-containing samples of 0.3 mL, and the samples were heated at 37°C for 5 minutes. 0.6



mL of Reagent B was added to each heated solution and the solution was reacted at 37°C for 10 minutes. The absorbance at 555 nm was measured and an increase in absorbance ( $\Delta OD$ ) after 10 minutes of reaction was determined. An example of the measurement result of  $\alpha$ -glycated dipeptides with various concentrations is shown in Fig. 1. Fig. 1 shows a linear correlation between  $\Delta OD$  and the  $\alpha$ -glycated dipeptide concentration. The  $\alpha$ -glycated dipeptide in a sample can be assayed in a short period of time with high accuracy. In contrast, instead of the oxidase of the present invention in Reagent B (oxidase reagent), 4 U/mL of the fructosyl amino acid oxidase (Japanese Patent Publication (kokoku) Nos. 33997/1993 (Hei5-33997) and 65300/1994 (Hei6-65300)) produced from a bacteria belonging to the conventional genus *Corynebacterium* was used to assay in the same manner as described above. None of the samples, however, exhibit the increase in absorbance ( $\Delta OD$ ). This indicates that modification of the conventional fructosyl amino acid oxidase has led to the provision of a novel oxidase of the present invention having activity that acts on a glycated peptide.

#### Example 5 (Assay of glycated protein using oxidase)

[0057] The following reagents were prepared for use in the assay of the glycated protein using the oxidase of the present invention.

Reagent A (coloring reagent)

Same as Example 4.

Reagent B (oxidase reagent)

Same as Example 4.

[0058]

Reagent C (protease reagent)	
Molsin (commercially available from Seishin Corporation)	20 mg/mL
Potassium chloride-hydrochloric acid buffer (pH 3.0)	100 mM

[0059] A nonglycated hemoglobin fraction and a glycated hemoglobin (HbA1c) fraction which were fractionated from human hemolysate in accordance with conventional methods (a combination of centrifugation, concentrating dialysis, ion exchange high performance liquid chromatography and the like) were mixed in a suitable ratio to prepare several types of samples with a HbA1c content (HbA1c value) of 0 to 50% based on the entire hemoglobin. 100  $\mu$ L of Reagent C (protease reagent) was added to 100  $\mu$ L of the sample and the sample was treated with protease at 37°C for 1 hour. The reaction mixture was then boiled to stop protease reaction. Subsequently, 0.5 M NaOH was added to the reaction mixture to adjust the pH value to 7, followed by centrifugation (12,000 rpm, 5 minutes) to fractionate the supernatant. 2.1 mL of Reagent A (coloring reagent) and 0.6 mL of Reagent B (oxidase reagent) were added to 0.3 mL of this supernatant, followed by mixing. The mixture was then subjected to reaction at 37°C for 30 minutes. The absorbance at 555 nm before the initiation of the reaction and the absorbance at 555 nm after the completion of the reaction were respectively measured to determine the increase in the absorbance ( $\Delta OD$ ). An example of the measurement result for several samples with different HbA1c values is shown in Fig. 2. This result shows a linear correlation between  $\Delta OD$  and the amount of HbA1c in the starting sample. Thus, glycated hemoglobin in a sample can be assayed in a simple and rapid manner with high accuracy.

#### INDUSTRIAL APPLICABILITY

[0060] The assay method of the present invention is effectively used in diagnosis or control of diabetic conditions by realizing the assay of a glycated protein, for example, glycated hemoglobin, in a short period of time, in a simple manner, and with high accuracy.

[0061] All publications, patents and patent applications cited herein are incorporated herein by reference in their entirety.

## Claims

- 5 1. A method for assaying the presence and/or amount of a glycosylated protein in a sample, wherein the sample is treated with protease, followed by treatment with an oxidase having an activity to produce hydrogen peroxide upon reacting with a glycosylated peptide to assay the presence and/or amount of a generated product or consumed substance by said reaction.
- 10 2. The method for assaying the presence and/or amount of a glycosylated protein according to claim 1, wherein the protease is at least one protease selected by proteases produced by microorganisms belonging to the genus *Aspergillus*, *Saccharomyces*, or *Bacillus*.
- 15 3. The method for assaying the presence and/or amount of a glycosylated protein according to claim 1, wherein the glycosylated peptide is an  $\alpha$ -glycosylated peptide.
- 20 4. The method for assaying the presence and/or amount of a glycosylated protein according to claim 3, wherein a peptide portion of the  $\alpha$ -glycosylated peptide is a short chain peptide having 2 to 6 amino acids.
- 25 5. The method for assaying the presence and/or amount of a glycosylated protein according to claim 3, wherein the  $\alpha$ -glycosylated peptide is fructosyl valyl histidine.
- 30 6. The method for assaying the presence and/or amount of a glycosylated protein according to claim 1, wherein the product to be assayed is hydrogen peroxide.
- 35 7. A method for assaying the presence and/or amount of a glycosylated protein in a sample, wherein the sample is treated with protease and the presence or absence, and/or amount of liberation of fructosyl valyl histidine is then assayed by HPLC.
- 40 8. A method for assaying the presence and/or amount of a glycosylated peptide in a sample, wherein the sample is treated with an oxidase having an activity to produce hydrogen peroxide upon reacting with the glycosylated peptide to assay the presence and/or amount of a generated product or consumed substance by said reaction.
- 45 9. A reagent kit for assaying a glycosylated protein in a sample, comprising the following components:
  - (i) protease;
  - (ii) an oxidase having an activity to produce hydrogen peroxide by reacting with a glycosylated peptide; and
  - (iii) a reagent for assaying hydrogen peroxide.
- 50 10. The reagent kit for assaying a glycosylated protein in a sample according to claim 9, wherein the glycosylated peptide is an  $\alpha$ -glycosylated peptide.
- 55 11. The reagent kit for assaying a glycosylated protein in a sample according to claim 10, wherein a peptide portion of the  $\alpha$ -glycosylated peptide is a short chain peptide having 2 to 6 amino acids.
12. The reagent kit for assaying a glycosylated protein in a sample according to claim 10, wherein the  $\alpha$ -glycosylated peptide is fructosyl valyl histidine.

Fig. 1

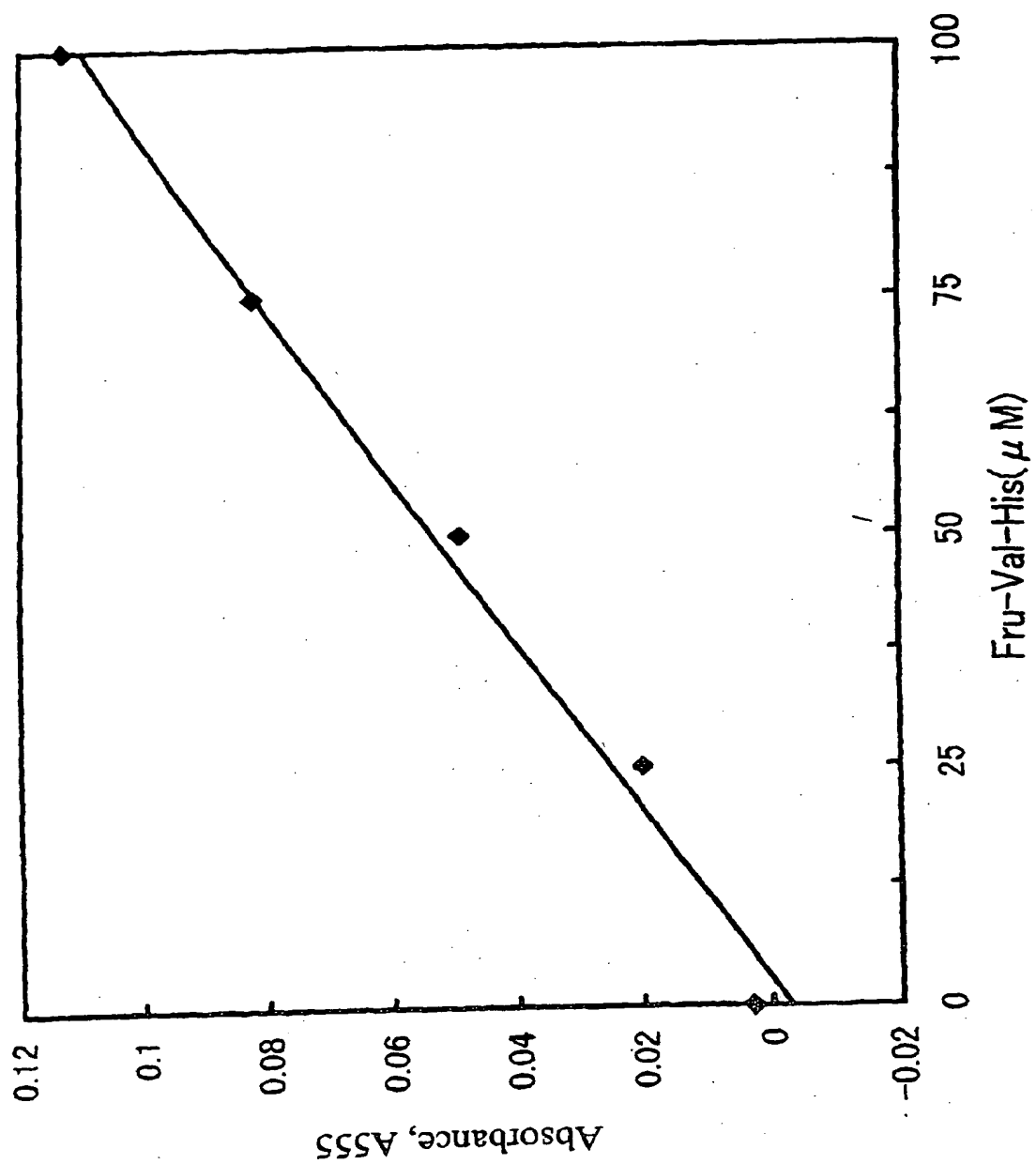
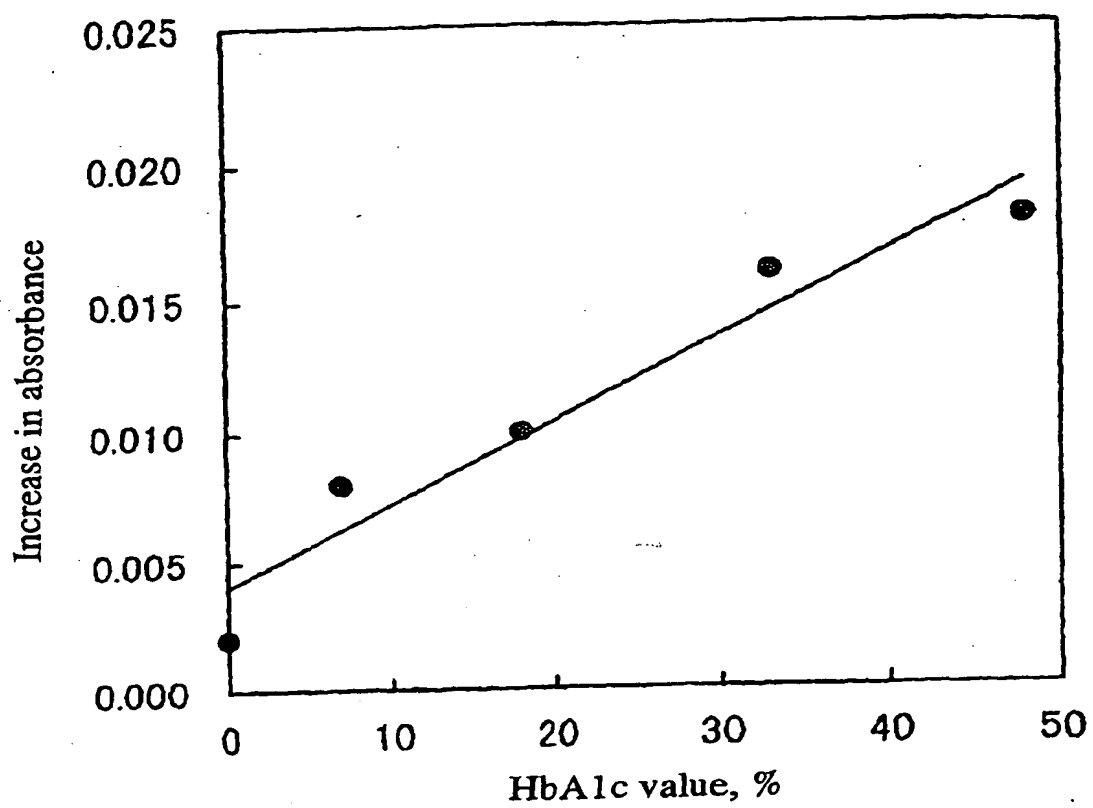


Fig. 2



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP00/06808

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> Int.Cl <sup>7</sup> C12Q1/26, C12Q1/37, G01N30/88		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) Int.Cl <sup>7</sup> C12Q1/26, C12Q1/37, G01N30/88		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) CA (STN), REGISTRY (STN), WPI (DIALOG), BIOSIS (DIALOG)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	WO, 00/50579, A1 (KDK CORPORATION), 31 August, 2000 (31.08.00) (Family: none)	1-12
X/Y/A	WO, 96/34977, A (GENZYME LTD), 07 November, 1996 (07.11.96) & JP, 11-504808, A & EP, 823943, A & US, 6008006, A	1,2/ 3,4,6-11/ 5,12
Y/A	EP, 693559, A (BOEHRINGER MANNHEIM GMBH), 24 January, 1996 (24.01.96) & JP, 8-62221, A & US, 5631140, A & DE, 4425162, A	3,4,6-11/ 5,12
A	EP, 598329, A (BOEHRINGER MANNHEIM GMBH), 25 May, 1994 (25.05.94) & JP, 6-225790, A & DE, 4310500, A	1-12
A	WO, 97/13872, A1 (KDK CORPORATION), 17 April, 1997 (17.04.97) & JP, 9-514821, A	1-12
A	EP, 526150, A (GENZYME CORP),	1-12
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 31 October, 2000 (31.10.00)		Date of mailing of the international search report 14 November, 2000 (14.11.00)
Name and mailing address of the ISA/ Japanese Patent Office		Authorized officer
Facsimile No.		Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

International application No.

PCT/JP00/06808

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	<p>03 February, 1993 (03.02.93)            &amp; JP, 5-192193, A &amp; US, 5370990, A</p>	

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## INTERNATIONAL SEARCH REPORT

International application No.

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**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

See extra sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

## INTERNATIONAL SEARCH REPORT

International application No.

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Continuation of Box No.II of continuation of first sheet(1)

The special technical feature of claims 1 to 6 and 8 to 12 resides in the point of comprising treating a sample with a protease, thus releasing glycopeptides, further treating the sample with an oxidase acting on these glycopeptides, and assaying the product thus formed.

The special technical feature of claim 7 resides in the point of assaying the released glycopeptides by HPLC.

The characteristic presented as a single general inventive concept in both of these groups of claims is the constitution of "treating a sample with a protease and assaying the thus released glycopeptides".

Because of being described in Clin. Chem., 43:1994-1951(1997), the above point is not a novel constitution. Such being the case, the present application does not comply with the requirement of unity of invention as specified in Rule 13 of the Regulations under the PCT.